# INVESTIGATING THE EXPRESSION AND FUNCTIONAL ACTIVITIES OF THE PROTEINS THAT ARE INVOLVED IN UTERINE FLUID PH REGULATION AT DIFFERENT STAGES OF THE OESTROUS CYCLE AND IN STEROID REPLACED OVARIECTOMIZED RATS

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## ABSTRACT

Precise control of the uterine luminal fluid pH is important for a number of key reproductive events. We hypothesized that sex-steroid could participate in this control via affecting the expression of $H^+$ and $HCO_3^-$ transporters as well as the enzyme that is involved in their generation. In view of this, we investigate the expressions and functional activities of few membrane transporters including CFTR, SLC26A6, NBC and NHE as well as CA enzyme in the uterus. Adult female WKY rats were divided into two main groups; (i) ovariectomized, steroid-replaced and (ii) intact rats at different phases of the oestrous cycle. The level of mRNA, the expression and distribution of proteins as well as the functional involvement of these transporters /enzyme were investigated.

Our functional studies revealed that estrogen caused an increase in the pH, volume, $Cl^-$, $HCO_3^-$ and $Na^+$ content of this fluid while progesterone resulted in *vice versa*. The effects of estrogen were antagonized by glibenclamide (CFTR inhibitor), DIDS (anion-exchanger inhibitor) and acetazolamide (CA inhibitor) while progesterone effects were inhibited by acetazolamide and EIPA (NHE inhibitor). A similar increase in the pH, volume, $HCO_3^-$, $Cl^-$ and $Na^+$ content were observed during estrus and proestrus stages, which were inhibited by glibenclamide, DIDS and acetazolamide. Meanwhile, acetazolamide and EIPA antagonized the reduction in pH, volume, $Cl^-$, $HCO_3^-$ and $Na^+$ concentrations during diestrus. The mRNA and protein expression of CFTR, SLC26A6 and NBC were increased following estrogen treatment while P treatment resulted in a decrease in these transporters expression. While NHE1 expression was up-regulated by P, NHE2 and NHE4 expressions were up-regulated by estrogen. Meanwhile, CAII and XII expressions were both stimulated by estrogen and progesterone although

progesterone treatment resulted in a lower expression of CA enzyme as compared to estrogen.

A similar effect was observed at different phases of the oestrous cycle, during which the pH and volume of the fluid were both increased. This was associated with an increase in the expressions of CFTR, NBC and SLC26A6. At metestrus and diestrus however, an increased in NHE1 expression together with the reduction in CFTR, NBC and SLC26A6 could result in the lower luminal fluid pH and volume.

Immunohistochemistry findings indicated that CFTR and SLC26A6 were expressed at the apical membrane, while NBC was bilaterally expressed with a higher intensity at the basolateral membrane under the estrogen effect and at estrus. NHE isoforms expression revealed bilateral localization of this protein's isoforms with NHE1 distributed mainly on the apical membrane under the influence of progesterone and during diestrus while NHE2 and NHE4 were distributed mainly at the basolateral membrane under estrogen influence and at estrus. CA II was expressed exclusively in the glandular epithelia while CAXII was expressed at both the glandular and endometrial epithelia.

In conclusion, the differential expression of these transporters/ enzyme by sex-steroids and at different phases of the oestrous cycle may explain the changes in the uterine fluid pH under these different conditions.

## ABSTRAK

Kawalan tepat pH cecair rongga rahim adalah penting bagi beberapa acara utama pembiakan. Kami menghipotesiskan bahawa seks steroid boleh menyertai dalam pengawalan cecair ini dengan mempengaruhi pengangkut H + dan HCO3- serta enzim yang terlibat di dalam penghasilan mereka. Berdasarkan pandangan ini, kami menyiasat expresi dan aktiviti fungsi beberapa pengangkut membran termasuk CFTR, SLC26A6, NBC dan NHE serta enzim CA di dalam rahim. Tikus WKY betina dewasa telah dibahagikan kepada dua kumpulan utama; (i) tikus yang yang ovariektomikan, serta dirawati dengan steroid dan (ii) tikus pada pelbagai fasa kitaran oestrous. Ekspresi mRNA dan protein/ enzim berserta penglibatan mereka di dalam fungsi pengangkutan ini telah disiasat.

Kajian fungsi kami mendedahkan bahawa estrogen yang menyebabkan peningkatan di dalam pH, isipadu serta kandungan $Cl^-$, $HCO_3^-$ dan $Na^+$ cecair ini manakala progesteron menyebabkan sebaliknya. Kesan estrogen telah dihalang oleh glibenclamide (perencat CFTR) dan DIDS (perencat anion-penukar) dan acetazolamide (perencat CA) manakala kesan progesteron dihalang oleh acetazolamide dan EIPA (perencat NHE). Peningkatan yang sama di dalam pH, isipadu serta kandungan $HCO_3^-$, $Cl^-$ dan $Na^+$ telah diperhatikan semasa peringkat estrus dan proestrus, yang direncat oleh glibenclamide, DIDS dan acetazolamide. Sementara itu, acetazolamide dan EIPA menghalang pengurangan pH, isipadu serta peningkatan kepekatan $Cl^-$, $HCO_3^-$dan $Na^+$ semasa peringkat diestrus. Ekspresi mRNA dan protein bagi CFTR, SLC26A6 dan NBC telah meningkat berikutan rawatan E manakala rawatan P mengakibatkan penurunan di dalam ekspresi pengangkut-pengangkut ini. Walaupun ekspresi NHE1 dikawal oleh P, ekspresi NHE2 dan NHE4 dikawal oleh estrogen. Sementara itu, ekspresi CAII dan XII kedua-duanya dirangsang oleh estrogen dan progesteron walaupun rawatan progesteron menyebabkan ekspresi enzim CA yang lebih rendah berbanding rawatan estrogen.

Satu kesan yang sama telah diperhatikan pada fasa yang berbeza kitaran oestrous, di mana pH dan isipadu cecair pada kedua-dua fasa meningkat. Ini telah dikaitkan dengan peningkatan di dalam ekspresi CFTR, NBC dan SLC26A6. Pada fasa metestrus dan diestrus bagaimanapun, peningkatan di dalam ekspresi NHE1 bersama dengan pengurangan di dalam CFTR, NBC dan SLC26A6 menyebabkan pH cecair berongga lebih rendah.

Penemuan immunohistokimia menunjukkan bahawa CFTR dan SLC26A6 telah diagihkan pada membran apikal, manakala NBC diaagihkan pada kedua-dua membran dengan intensiti yang lebih tinggi pada membran basolateral di bawah kesan estrogen serta pada fasa estrus. Ekspresi isoforms NHE mendedahkan pengagihan dua hala isoforms ini dengan protein NHE1 diagihkan terutamanya pada membran apikal di bawah pengaruh progesteron dan semasa fasa diestrus manakala NHE2 dan NHE4 diagihkan terutamanya pada membran basolateral bawah pengaruh E dan pada fasa estrus. CA II telah diekspresi secara eksklusif di dalam epithelia kelenjar rahim sementara CAXII telah diagihkan di kedua-dua epithelia kelenjar rahim serta lapisan endometrium.

Kesimpulannya, ekspresi berbeza pengangkut / enzim oleh seks steroid dan pada pelbagai fasa kitaran oestrous boleh menjelaskan perubahan di dalam pH bendalir rahim di bawah pengaruh hormon yang berbeza.

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| | |
|---|---|
| ACTZ | Acetazolamide |
| APS | Ammonium Persulphate |
| AMRC | apical mitochondria-rich cells |
| AE | Anion exchanger |
| AQP | Aquaporin |
| AR | Androgen receptor |
| BCA | Bicinchoninic |
| BSA | Bovine Serum Albumin |
| CA | Carbonic Anhydrase |
| CFTR | Cystic Fibrosis Transmembrane Conductance Regulator |
| CF | Cystic Fibrosis |
| DAB | Diaminobenzidine |
| DIDS | 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS) |
| Ds | Diestrus |
| E | estradiol/estrogen |
| EIPA | 5-(N-Ethyl-N-isopropyl)amiloride |
| ENaC | epithelial $Na^+$ channel |
| ER | Estrogen receptor |
| Es | Estrus |
| G | Glibenclamide |
| HSP | Hydrosalpinx |
| IHC | Immunohistochemistry |
| IGF | Insulin like Growth Factor |
| IP | Intraperitoneal |
| ISE | Ion Selective Electrode |
| Ms | Metestrus |
| NBC | Sodium-Bicarbonate Cotransporter |
| NHE | Sodium- Hydrogen Exchanger |
| OHSS | Ovarian hyperstimulation syndrome |
| OVX | Ovariectomized |
| P | Progesterone |
| PBST | Phosphate-Buffered Saline/Tween |
| PG | Prostaglandin |
| pHi | Intracellular pH |
| PKA | Protein kinase A |
| PR | Progesterone receptor |
| PP | Proton Pump/ $H^+$ ATPase |
| Ps | Proestrus |
| PVDF | Polyvinylidene difluoride |
| qPCR | Quantitative real time PCR |
| SDS | Sodium Dodecyl Sulphate |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| WB | Western blotting |

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Chapter 1

# INTRODUCTION

## 1.1 Introduction

The precise regulation of uterine fluid pH is important for a number of key reproductive events including sperm transport and capacitation, fertilization, embryo transport and implantation (Aguilar & Reyley, 2005; Boatman, 1997). The exact pH of the uterine fluid at different phases of the rodent's oestrous cycle is currently unknown; however, limited data indicate that the pH is high at around the time of ovulation (Blandau, *et al.*, 1958). Sex-steroids in particular estrogen (E) and progesterone (P) may be involved in the regulation of uterine fluid pH since they have found to affect other uterine fluid parameters such as volume and electrolyte content (Naftalin, *et al.*, 2002; Salleh, *et al.*, 2005). The evidence that sex-steroid may affect uterine fluid pH comes from an observation that the uterine fluid $HCO_3^-$ content is high under the influence of E (Murdoch & White, 1971; Vishwakarma, 1962).

$HCO_3^-$ and $H^+$ are the two most important determinants of body fluid pH. Factors affecting their secretion or generation can thus affect the pH of body fluid. $HCO_3^-$ secretion may involve multiple membrane proteins including cystic fibrosis transmembrane conductance regulator (CFTR) (Wang, *et al.*, 2003a), sodium bicarbonate cotransporter (NBC) (Wang, *et al.*, 2002) and $Cl^-/HCO_3^-$ exchanger (SLC26A6) (Jensen, *et al.*, 1999b) while $H^+$ secretion may involve the sodium proton exchanger (NHE) (Bagnis, *et al.*, 2001) and $H^+$-ATPase pump (Herak-Kramberger, *et al.*, 2001). In addition, the carbonic anhydrase (CA) enzyme may participate in $HCO_3^-$ and $H^+$ synthesis (Lutwak-Mann, 1955). Apart from the well documented involvement of CFTR in mediating endometrial $HCO_3^-$ secretion (Chan, *et al.*, 2002; Salleh, *et al.*, 2005), the participation of other endometrial proteins in uterine fluid pH regulation have not been well documented.

CFTR is a $Cl^-$ channel expressed in the apical membrane of the epithelia and responds to an increase level of intracellular cyclic AMP (cAMP) by secreting $Cl^-$ (Bargon, *et al*;

1992). Additionally, CFTR has also been shown to mediate cAMP dependent $HCO_3^-$ secretion in the endometrial epithelia (Wang, *et al.*, 2003a). Its expression is up-regulated by E and at proestrus and estrus phases of the oestrous cycle (Chan, *et al.*, 2007; Rochwerger & Buchwald, 1993) and down-regulated by P (Mularoni, *et al.*, 1995).

SLC26A6, from the $Cl^-$/ $HCO_3^-$ exchanger family, is expressed on the luminal membrane of secretory epithelia. SLC26A6 has been reported to be involved in the regulation of intracellular fluid pH and cell volume via extrusion of $HCO_3^-$ into the lumen in exchange for $Cl^-$ (Jensen, *et al.*, 1999b). The effect of sex-steroids on its expression and functional activities is not fully understood.

NHE, on the other hand consists of nine isoforms, NHE1-9 (Orlowski & Grinstein, 2004) and is involved in pH regulation of by extruding $H^+$ in exchange for $Na^+$ (Dunham, *et al.*, 2004). It is involved in intracellular pH homeostasis (Wang, *et al.*, 2003b). The involvement of NHE in intracellular pH regulation has been reported in the epididymis (Kaunisto & Rajaniemi, 2002) and pancreas (Brown, *et al.*, 2003). NHE expression has been reported in the endometrium (Salleh, *et al.*, 2011), however the changes in its expression and functional activity under the influence of sex-steroid has not been reported.

In addition to NHE that regulates the intracellular pH (pHi), Fong (1998b) has shown that $HCO_3^-$ secretion depends substantially on basolaterally situated $Na^+$-$HCO_3^-$ cotransporter (NBC). NBC has been implicated in the regulation of intracellular pH and epithelial $HCO_3^-$ secretion (Yamaguchi & Ishikawa, 2008). Functional studies by Wang *et al*, (2002) has revealed that the $pH_i$ recovery was observed only when both $Na^+$ and $HCO_3^-$ were present in the basolateral compartment which suggested that $HCO_3^-$ influx through the basolateral membrane requires the presence of $Na^+$. These results point to the involvement of NBC in cellular pH content. The presence of NBC in the mouse

endometrial epithelium was furthermore, confirmed by the results of RT-PCR (Wang, *et al.*, 2002). Despite this information the pattern of NBC protein and mRNA expression at different stages of the estrous cycle and under the effect of exogenous steroid remains unknown.

CA, a zinc containing metalloenzyme with 16 isoforms, is involved in the synthesis of $H^+$ and $HCO_3^-$ via reversible hydration of $CO_2$ (Boron, 2010). It is the main enzyme that contributes to acidification of epididymal fluid and urine (Breton, *et al.*, 1996), and alkalinization of the pancreatic fluid (Sly & Hu, 1995). The expression of different CA isoforms has been reported in human, rat and mouse endometrium (Ge & Spicer, 1988; Sly & Hu, 1995). The effect of sex-steroids on CA expression and functional activity is still unknown.

It is hypothesized that uterine fluid pH fluctuates throughout the oestrous cycle and it is under the control of sex-steroids. We further hypothesize that the effect of sex-steroids on the pH changes is mediated via multiple endometrial proteins such as CFTR, SLC26A6, NHE, NBC and CA. In view of this, the aim of the study was to investigate the involvement of these proteins in uterine fluid pH changes under the influence of sex-steroids and at different phases of the oestrous cycle. In parallel, the changes in other parameters such as uterine fluid volume and electrolytes ($HCO_3^-$, $Cl^-$ & $Na^+$) content were also investigated under a similar hormonal condition.

The general objective of this study is to investigate the influence of ovarian hormones in the changes of uterine milieu.

The specific objectives of the study are:

1- To investigate the effect of E and P on uterine fluid pH, volume and ionic content in steroid replaced ovariectomized rats and at different stages of the oestrous cycle.

2- To evaluate the role of CA in uterine fluid changes by the application of acetazolamide in uterine perfusion in various experimental groups.

3- To assess the contribution of CFTR to uterine fluid content by introduction of glibenclamide.

4- To assess the involvement of other bicarbonate transporters via inhibitory effect of DIDS on uterine fluid.

5- To investigate the importance of NHE in uterine fluid changes by the application of EIPA.

6- To evaluate the mRNA level, protein expression and localization of CAs, CFTR, NHEs, SLC26A6 and NBC in steroid treated rats and throughout the oestrous cycle.

Chapter 2

# Literature review

## 2.1 Endometrium

The uterus plays a major role in reproductive biology of mammals by providing a suitable environment for the embryo and fetal development. The uterus achieves this important function by synthesis and secretion of different products through uterine endometrium (Davis & Blair, 1993) (Figure 1).

The endometrium is uterine epithelia which is exposed to E and P cyclically and undergoes different changes consisting of proliferation, differentiation and menstruation in human and some mammals. The endometrium is capable of shedding and regeneration in the absence of pregnancy; hence it can be considered a site of physiological injury and repair (Ollo, 1991).

### 2.1.2 Endometrial structure

The endometrium consists of two layers: upper functional layer of columnar epithelia, which is shed at menstruation and an underlying thick connective tissue, stroma responsible for regeneration of the endometrium, and vary in thickness according to hormonal influence (Lutz, 2009). The endometrium consists of different cell types, luminal and glandular epithelium and endometrial stroma (Beier & Beier-Hellwig, 1998; Davis & Blair, 1993), (Figure 1.c).

Before puberty endometrium consist of tubular gland, dense fibroblast in stroma and thin blood vessels. Upon activation of the hypothalamus-pituitary-gonadal axis, the endometrium undergoes cyclic morphological changes. Under E dominance, endometrium proliferates. The endometrial glands grow and become tortuous (proliferative phase). After ovulation, P secretion inhibits the proliferation and induces a complex of secretory activity. These secretory changes occur in the E primed endometrium (Deligdisch, 2000).

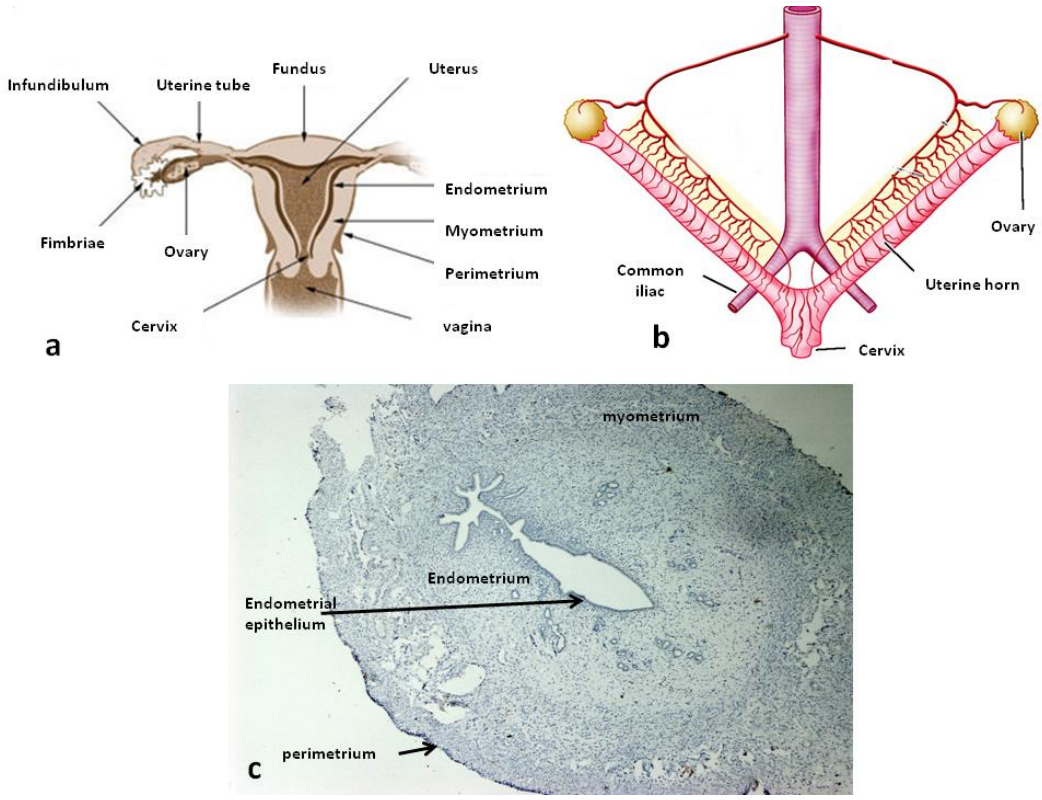



**Figure 2. 1** Human uterine structure and histology in (a) which shows endometrium, myometrium and perimetrium. In rat, uterus formed of two horn (b) and endometrium, myometrium and perimetrium can be seen in horizontal section of rat uterus under microscope (c).

**Source**: a (Kim, *et al.*, 2009), and b (Osol & Mandala, 2009)

### 2.1.3 Endometrium changes under the effect of ovarian hormone

The effect of sex-steroid on the uterus is via their respective hormone receptors that initiate a cascade of gene expression involved in implantation and early pregnancy. E is responsible for proliferative changes during follicular phase of the ovarian cycle, while P leads to differentiation during the luteal phase. The effect of P relies on the existence of progesterone receptor (PR), while the presence of PR is depends on prior exposure of the endometrium to E. In rodents, E leads to several morphological changes such as increased depth of tight junction, flattening of microvilli and alteration in the apical cytoskeleton during embryo attachment (Nimbkar-Joshi, *et al.*, 2012). Concurrently

studies in monkey shows that these changes are probably mediated by up regulation of estrogen receptors α (ERα) and down regulation of ERβ (Nimbkar-Joshi, *et al.*, 2012).

### 2.1.4 Expression of steroid receptor in endometrium

Different protein receptors are expressed in the endometrium such as ER α and β; G protein coupled ER, known as G protein coupled receptor 30 (GPR 30), a non-nuclear receptor with high affinity binding site for E (Samartzis, *et al.*, 2012).

PR, includes two isoform PRA and PRB which is encoded by the same gene (Conneely & Lydon, 2000). The endometrium also expresses androgen receptor (AR), hence it either acts directly via its receptor or indirectly through ER after aromatization to E (Horie, *et al.*, 1992) and it is involved in anti-proliferative effect induced by anti-progestin (Brenner, *et al.*, 2002; 2003), (Table 1).

.

**Table 2. 1** Expression of steroid receptor in endometrium. (Adapted from Brenner, *et al.*, 2002; Horie, *et al.*, 1992; Henderson, *et al.*, 2003)

| | **Estrogen receptor (ER)** | | **Progesterone receptor (PR)** | **Androgen receptor (AR)** | **Glucocorticoid receptor (GR)** |
|---|---|---|---|---|---|
| | ERα | ERβ | | | |
| **Endometrium Functional layer Upper zone** | - Increase in glandular and stroma in proliferative phase<br>- Decrease in secretory phase (due to progesterone) | Decline in late secretory phase | Reduce in gland during transition from proliferative to secretory phase, highly express in stroma | - Up regulated in estrogen proliferative phase<br>- Down regulated in secretory phase<br>-Minimal expression in gland.<br>Mostly in Stroma | -Expressed throughout the cycle<br>The function is not clear |
| **Endometrium Basal layer** | In glandular and stroma across the cycle | | In glandular and stroma express throughout the cycle | | |

## 2.2 Uterine fluid

The endometrium plays an important role in the early stages of embryonic life, embryo relationship with mother, and in pregnancy through the secretion of uterine fluid (Bauersachs, *et al.*, 2005). This fluid of the uterine lumen is essential for sperm and embryo transport, development and implantation. Even though the surface epithelial cells are capable of producing fluid but, the main source of the fluid in the endometrium are the glandular cells.

Uterine fluid contains hormones, prostaglandin (PG), enzymes, energy substrates, ions, vitamins, amino acids, peptides, serum proteins and uterine proteins. These are essential for oocyte and spermatozoa movement, capacitation of the spermatozoa and implantation (Yang, *et al.*, 2004), early embryonic development (Aguilar & Reyley, 2005; Wang, *et al.*, 2003a), and embryo viability (Shixiong, 2000). The protein pattern, ionic composition and volume of this fluid varies during the stages of the cycle (Abuladze, *et al.*, 1998; Casslén & Nilsson, 1984). The exchange rate of the fluid between vascular and extra vascular fluid is determined by the uterine blood barrier; whereby extra vascular fluid is divided into vascular, endometrial extracellular and luminal extracellular fluid (McRae, 1988; McRae & Heap, 1988).

### 2.2.1 Uterine fluid composition

#### *2.2.1.1 Amino acids and proteins*

There are 25 amino acids in the tubal fluid of cows, ewes, pigs, rabbits and mice. Among all the amino acid glycine has the highest concentration in all species. It protects preimplantation of mouse embryos against the detrimental effect of inorganic ions (Van Winkle, *et al.*, 1990). Free amino acids are important for the survival of gamete and embryo, and are varied between species and during stages of the oestrous cycle (Aguilar & Reyley, 2005). Protein in tubal fluid is lower than protein in plasma and its content is

about 10-15% of serum protein content. Most of it consists of albumin and immunoglobulin G derived from blood serum. Total protein concentration of uterine fluid is associated with ovarian hormone cycle, for example in the bovine uterus, protein profile changes during the oestrous cycle in term of quality and quantity, and may prepare the uterus for the physiologic events (Alavi-Shoushtari, *et al.*, 2008). The amount of tubal glycoprotein is affected by stages of the oestrous cycle (Abe & Abe, 1993; Aguilar & Reyley, 2005; Kapur, 1988), and has the highest level during the periovulatory period (Abe, *et al.*, 1998; Aguilar & Reyley, 2005; Leese, 2001). Moreover, it has been proved that E induces the synthesis and secretion of oviduct-specific glycoprotein in the golden hamster while P has a lesser effect (Abe, *et al.*, 1998; Aguilar & Reyley, 2005; Leese, 2001; Sun, *et al.*, 1997).

#### *2.2.1.2 Prostaglandins*

PG level in the uterus is under the influence of ovarian hormones and it fluctuates throughout the stages of the oestrous cycle. In pigs, for example, the highest amount of PGF2α was detected on the second day of standing heat (Rodriguez-Martinez, *et al.*, 1983). In rabbit PGE and F significantly increases before ovulation which could be related to ovum capture (McComb, 1985). In human, PG level varies at different region of the oviduct and at different stages of the cycle. Lower level of PGE and PGF is detected at the utero-tubal junction, while the highest amount is in fimbriae. At luteal phase their concentration is two times more than in the follicular phase (Nieder & Augustin, 1986). Furthermore, in human tubes the number of binding sites for PGE2 and F2 are greater in the isthmus and in the luteal phase (Sato, 1988).

#### *2.2.1.3 Growth factors*

Several growth factors participates in early embryogenesis, trophoblast growth, endometrial cell differentiation, conceptus invasiveness and regulation of

steroidogenesis (Simmen & Simmen, 1991). In addition, growth factor levels vary during the cycle; in pig, for instance, IGF-I and II are in greater amount at estrus than pre- or postestrus, even though their concentration is similar at both stages (Wiseman, *et al.*, 1992).

#### *2.2.1.4 Ionic content of the uterine fluid*

Ions are involved in different biochemical functions in the reproductive system. *In vitro* studies have revealed that cations contribute to acrosome reaction, sperm motility, capacitation and fertilization (Grippo, *et al.*, 1992). $Ca^{+2}$ for instance has a major role in sperm capacitation, while $Na^{+}$ and $K^{+}$ are necessary for zona-induced acrosome reaction (Grippo, *et al.*, 1992).

In the female reproductive tract, ionic composition is similar to that of serum, and it may be affected by stages of the reproductive cycle (Deachapunya & O'Grady, 1998; Matthews, *et al.*, 1998; Nichol, *et al.*, 1992). In addition steroids can regulate the ion flow through the oviductal epithelium similar to the regulation of ions flow in trachea cells (Leese, 2001; Zeitlin 1989) and in kidney tubule (Leese, 2001; Verlander, 1998).

$K^{+}$ content in human oviduct and uterus, and bovine oviduct is higher than in plasma (Deachapunya & O'Grady, 1998; Matthews, *et al.*, 1998; Schul, *et al.*, 1971). However, $Ca^{+2}$ and $Na^{+}$ in cow and human are in lower concentration in the uterus than blood (Matthews, *et al.*, 1998; Schul, *et al.*, 1971). $Ca^{+2}$ concentration increases during ovulation in the isthmus region of cow oviduct and is more than $Ca^{+2}$ concentration in plasma (Grippo, *et al.*, 1995). In cow, $Mg^{+2}$ concentration is significantly affected by different stages of the oestrous cycle, and it is lower than plasma level. In mare however $Mg^{+2}$ is 2-5 times higher than its plasma concentration which is also much more higher than what is measured in other species (Aguilar & Reyley, 2005; Grippo, *et al.*, 1992). Table 2.2 shows the concentration of ions in the tubal fluid in some species.

**Table 2. 2** Ionic concentration of uterine fluid in different species from different studies has reviewed by Aguilar *et al.* ( Adapted from Aguilar & Reyley, 2005).

| | Cow | Rabbit | Rabbit | Human | Human | Mare |
|---|---|---|---|---|---|---|
| $Na^+$ | 86.1 | 127.8 | 189.7 | 130 | 145 | 129.5 |
| $Cl^-$ | 112.7 | 115.4 | 332.2 | 132 | 119.5 | |
| $K^+$ | 65.7 | 5.6 | 16.8 | 21.1 | 6.7 | 7.9 |
| $Ca^{+2}$ | 3.19 | 7.98 | 2.71 | 1.13 | | 2.28 |
| Mg+2 | | 0.141 | 0.473 | 1.42 | | 4.59 |
| S | | | | 123 | | |
| P | 3 | 0.193 | | 8.69 | | 0.366 |
| Zn | | 0.099 | 0.0046 | | | |
| $HCO_3^-$ | | | 16.55 | | | |

In spite of the high $K^+$ concentration of tubal fluid, the total cation concentration in the uterine fluid is less than the serum cation concentration (Casslen & Nilsson, 1984; Matthews, *et al.*, 1998) due to active absorption of $Na^+$ and $Ca^{+2}$ and passive $K^+$ secretion. This phenomena have occurred in primary monolayer of human endometrial epithelium (Matthews, *et al.*, 1998) and in the uterus of immature pig (Chan, *et al.*, 1997; Fong, *et al.*, 1998; Vetter & O'Gary, 1996). Thus, there is a high $K^+$: $Na^+$ ratio ($K^+$ 20mM and $Na^+$130mM) in rat and human uterine fluid under the influence of ovarian steroid hormones (Casslen & Nilsson, 1984; Clemeston, *et al.*, 1970).

It has been reported that $Cl^-$ concentration is not significantly different from serum concentration (Casslen & Nilsson, 1984), but the bicarbonate concentration was found to be two- to four fold higher than plasma (Murdoch & White, 1968, 1971). Moreover, $HCO_3^-$ concentration not only changes along the tract and depends on reproductive events but varies at different stages of the cycle and pregnancy, (Chan, *et al.*, 2006). This indicates that active bicarbonate transport across endometrium (Wang, *et al.*,

2003a) is presumably by NBC in the basal membrane (Wang, *et al.*, 2002), or CFTR and $Cl^-/HCO_3^-$ exchanger in the apical membrane (Wang, *et al.*, 2003a).

In spite of the above mentioned studies, Hugentobler, *et al* (2007) reported that there is no association between the ionic concentration in bovine oviduct and uterus with E and P levels. Differential regulation of ion secretion accounts for different concentration during the reproductive cycle.

### 2.2.2 Secretion of uterine fluid

The ionic concentration of the uterus (Chan, *et al.*, 1997; Nordenvall, *et al.*, 1989), and fluid volume (Parmar, *et al.*, 2008), are under the influence of steroid hormones. The highest amount of fluid accumulates during the proestrus phase of the rodent reproductive cycle (Huang, *et al.*, 1999). E predominantly stimulates tubal fluid secretion while P inhibits it (Aguilar & Reyley, 2005; Clemeston, *et al.*, 1970). Hence, fluid secretion increases at estrus and declines at diestrus and pseudopregnancy (Aguilar & Reyley, 2005; Dickens & Leese, 1994). In the rabbit amount of fluid production is higher in estrus than pseudopregnancy (Downing, *et al.*, 1997); whereas in monkey, fluid secretion is at its peak at ovulation (Brenner & West, 1975). In cows, the rate of oviductal fluid production are approximately 0.2ml and 2.0 ml per day at diestrus and estrus respectively (Schul, *et al.*, 1971). This means at estrus the oviduct presents the highest volume of fluid (Aguilar & Reyley, 2005; Leese, 2001). It has been reported that secretory rate of oviduct in ovariectomized ewe and rat is lower than the constant state at the luteal phase (Leese, 2001; Perkins, 1974). There is a great increase in secretion in ovariectomized ewe after treatment with E (Leese, 2001; Murray, 1995). However, the mechanism underlying this effect of ovarian steroid hormones has not been completely resolved (Leese, 2001).

### 2.2.3 Mechanism of fluid secretion

It is likely that fluid secretion is related to ionic flow, and ionic flow occurs prior to fluid movement in the epithelial cells (Chan, *et al.*, 2002). In secretory epithelia, the driving force for water movement is $Cl^-$ ion flow from the basal to the apical side (Downing, 1997; Leese, 2001; Quinton, 1990). In the apical membrane of epithelium, fluid absorption and secretion is dependent on $Na^+$ and $Cl^-$ conductance. Active $Na^+$ absorption leads to $Cl^-$ and fluid efflux from the lumen into the blood, while active secretion of $Cl^-$ results in $Na^+$ and fluid flow into the lumen (Chan, *et al.*, 2002). The $Na^+$–$K^+$ATPase, are responsible for the lower concentration of intracellular $Na^+$, and at the basolateral membrane the $Na^+$-$K^+$-$Cl^-$ cotransporter are responsible for the uptake of $Cl^-$, and due to the permeability of apical surface to $Cl^-$, anion moves down the electrochemical gradient into the lumen. Ion flow produces an electrical force that drives $Na^+$ paracellularly through the tight junctions between the cells. It is assumed that in secretory epithelia, localized accumulation of ions in the intracellular space causes high osmotic pressure. Water movement occurs to balance the osmotic equilibrium so that fluid accumulates in the lumen (Leese, 2001; Quinton, 1990).

At estrus, the height of epithelial cells increase to enhance the area of intercellular space available for localized accumulation of ions (Leese, 2001; Murray, 1995) and fluid production is at maximal level. Moreover, during estrus, glycoprotein production and secretion increases, which in turn enhances the viscosity of luminal fluid. It may also prevent rapid diffusion by accumulation of ions into microenvironments with high osmolarity (Hunter, 1994; Leese, 2001).

In the uterus, fluid absorption is under the influence of P via an amiloride sensitive channel (Salleh, *et al.*, 2005). It has been suggested that epithelial $Na^+$ channel (ENaC) is involved in fluid absorption, as the expression of ENaC subunits are stimulated by P. The level of ENaC mRNA in the uterus vary at different stages of the oestrous cycle

(Chan, *et al.*, 2002) and the highest expression is around the time of implantation (Yang, *et al.*, 2004). Thus due to the activity of ENaC and CFTR in the female reproductive tract, the ovary and oviduct are involved in fluid secretion while vagina and cervix contribute to fluid absorption. In the uterus both absorptive and secretory activities have been detected (Chan, *et al.*, 2002).

#### *2.2.3.1 Aquaporin (AQP) role in fluid secretion*

Transepithelial osmotic water flow results from transcellular ion secretion. Water crosses the membrane through the membrane lipid bilayer and paracellular junction. In addition water channels, the aquaporins (AQP) facilitate water flux. The AQP family consists of 13 members (Lee, *et al.*, 2012) and some of these AQP are expressed in the uterus.

AQP 1 that has been detected in the myometrium of mouse uterus is regulated by ovarian hormones. E in epithelial cell and myometrium stimulate AQP2 expression. AQP3 is expressed at low level in epithelial cells and myometrium. Water flux into the mouse uterine lumen is facilitated by both AQP2 and AQP3 (Jablonski, *et al.*, 2003).

### 2.2.4 Effect of growth factor and adrenaline on uterine fluids

Ovarian steroid hormones exert many of their actions through growth factors (such as IGF-1) and cytokines in the uterus. Katagiri, *et al* (1997) has reported that hyperestrogenemia due to superovulation increased the total cation content and uterine fluid volume by more than twofold. Percentages of $K^+$ and $HCO_3^-$ content to total cations or anions were enhanced by 27% and 16%, respectively, and $Na^+$ and $Cl^-$ content were attenuated by 26% and 15% respectively after superovulation. In this condition IGF-1 is an E mediator.

The effect of other factors such as adrenaline on uterine fluid composition has been studied before. Anion secretion in cultured mouse endometrial epithelium (Fong, *et al.,* 1998b) and in rat (Levin & Phillips, 1983) can be regulated by adrenaline via β adrenoceptors through a pathway that involves cAMP (Fong, *et al.,* 1998b). The effect of adrenaline could be inhibited by $Cl^-$ inhibitor, Diphenylamine-2, 2'-dicarboxylic acid, glibenclamide (Fong, *et al.,* 1998b) and DIDS, which are also inhibitors of $Ca^{+2}$ activated $Cl^-$ channels (Chan, *et al.*, 1997; Fuller & Benos, 1992). Replacement of external $Cl^-$ and $HCO_3^-$ but not amiloride or $Na^+$ replacement in apical solution is also able to inhibit the adrenaline effect. In spite of this, Levin and Scragill (1987) reported that only $HCO_3^-$ secretion is under adrenaline stimulation. In the inner medullary collecting duct cells (in kidney) of rat, cAMP induced cell acidification via anion secretion that is independent of acid secretion and is the result of activation of one or more $HCO_3^-$ exit pathways (Zhang, *et al.*, 1996).

The influence of cAMP on $HCO_3^-$ secretion across the endometrial epithelium, and dependence of some neurohormonal regulatory pathway on cAMP, suggests that probably $HCO_3^-$ content of the uterine is under precise neurohormonal regulation (Wang, *et al.*, 2002).

$PGF_{2\alpha}$ stimulates $Na^+$ absorption and $K^+$ secretion in the intact endometrium of pig (Chan, *et al.*, 1997; Vetter & O'Gary, 1996), however it has been previously reported that endometrium active secretion of $K^+$ and anion along with $Na^+$ absorption occurs in pigs (Chan, *et al.*, 1997; Deachapunya & O'Grady, 1998).

### 2.2.5 The role of $HCO3^-$ in uterine fluid

Bicarbonate is a biologic pH buffer that maintains acid-base homeostasis and protects the body against toxic flow from intra and extracellular pH fluctuation. In addition it

facilitates solubilisation of macromolecule such as mucin (Park & Lee, 2012; Roos & Boron, 1982). $HCO_3^-$ is involved in different physiologic events in the reproductive system. In the male reproductive tract, for example, low $HCO_3^-$ is the main reason of sperm being immotile, whereby during ejaculation high $HCO_3^-$ content of seminal plasma enhances sperm motility (Ekstedt, *et al.*, 2004) via activation of adenylate cyclase (Okamura, *et al.*, 1985). In the female reproductive tract $HCO_3^-$ contributes to capacitation and acrosome reaction. Furthermore, zone penetration by spermatozoa increase in comparison with bicarbonate free media (Amitabha & Yanagimachi, 1988). Bicarbonate participates in cleavage, embryonic formation particularly in blastocyst stage, and may be involved in pH regulation whereby low pH is harmful such as in hydrosalpinx (Karhumaa, 2002) .

$CO_2/HCO_3^-$ is the major physiological buffer that is responsible for pHi regulation. In this system, $CO_2$ usually can cross the membrane without involvement of protein transporters although aquaporin is able to enhance $CO_2$ movement. However $CO_2$ flux must be matched with $HCO_3^-$, thus the role of bicarbonate transporter is inevitable (Swietach, *et al.*, 2010).

### 2.2.6 Uterine fluid pH

Bicarbonate is the main ion involved in pH alteration. The main function of the vaginal and cervical epithelial cells is to regulate pH in the lumen of lower genital tract in female. pH is maintained between 4.5 and 6 during premenopausal years with mild alkalinization to about 6.5 before ovulation (Gorodeski, 2004).

pH in the female reproductive tract is affected by location and stages of the menstrual cycle. In the human vagina, for example, pH is acidic during the whole menstrual cycle, and seminal plasma increases vaginal pH form 4.3 to 7.2. This is due to high bicarbonate concentration. However in cervical mucus (6.5-7.5) and oviduct (7.0-8.0), pH is affected by stages of the cycle and the highest amount has been recorded at

ovulation in the cervix (Macdonald & Lumley, 1970). The pH of the uterus is unknown, thus it will be evaluated in this study in rat uterus as an animal model.

### *2.2.6.1 Physiologic role of uterine fluid pH*

The pH of uterine fluid is important for gamete transportation, sperm capacitation, and embryo development. It has been shown that culture of early bovine embryos at pH values less than 7 reduces cleavage rates and development to the blastocyt stage (Ocon & Hansen, 2003). Furthermore, pHi is the main parameter in cell homeostasis and contributes to a wide range of process such as biochemical reaction, channel/transport function and protein- protein interaction (Boron, 2010).

### *2.2.6.2 pH regulation*

Nichol, *et al* (1997) reported that the pH of oviductal fluid is controlled by both systemic and local mechanisms, and in pig the ipsilateral ovary and (or) embryonic factors influence the pH profile of the oviduct. In dairy cows, a short time increase in plasma urea nitrogen can decrease the pH in uterus (Rhoads, 2004).

The pH may change under the influence of steroid hormones (Pybus & Onderdonk, 1999). In rat or mouse pH is higher in the E replaced animal in comparison with control animals (He, *et al.*, 2010; Salleh, *et al.*, 2011). In addition in women, oral administration of E lead to a subtle alkalinization of the cervical mucus (Eggert-Kruse, 1993). E replacement after menopause decreases vaginal luminal pH from 6.5-7 to 5.5 which is due to active proton secretion into the vaginal lumen by vaginal ectocervical cells or presence of lactobacillus (Gorodeski, 2004). It seems that E has a different role in vagina in respect to other part of female genital tract.

### 2.2.7 Channels involved in $HCO_3^-$ transport

Accumulation of luminal $HCO_3^-$ in body cavities is mediated by basolateral membrane transporters that absorb $HCO_3^-$ from the blood; and apical membrane transporter that secrete $HCO_3^-$ into the luminal space.

Carbonic anhydrase (Cohen, *et al.*, 1976; Goyal, *et al.*, 1980), apical NHE3 (Bagnis, *et al.*, 2001; Kaunisto & Rajaniemi, 2002), cytoplasmic $H^+$/ATPase (Herak-Kramberger, *et al.*, 2001) in addition to basolateral $Cl^-$/ $HCO_3^-$ exchanger (AE2) (Jensen, *et al.*, 1999b) are involved in the regulation of pH in male reproductive tract.

In kidney proximal tubules, net $HCO_3^-$ reabsorption is achieved through apical $H^+$ secretion into the lumen via NHE3 and proton pump (PP). Then $H^+$ combines with $HCO_3^-$ via CA IV and produces $CO_2$ that later diffuses into the cell and it is converted to $H^+$ and $HCO_3^-$ by application of CAII. $HCO_3^-$ diffuses passively through the basolateral NBC. In the thick ascending limb $HCO_3^-$ reabsorption is mediated by $Cl^-$/ $HCO_3^-$ exchanger (AE1) (Breton, 2001).

It has been suggested that these channels contribute to changes in uterine fluid pH. This fact is observed through regulation of uterine intracellular pH ($pH_i$) by NHE, $Na^+$, $HCO_3^-$/$Cl^-$ exchanger and $Cl^-$/ $HCO_3^-$exchanger, whereby the first two channels increase $pH_i$ whereas the latter decreases it (Dale, *et al.*, 1998).

The activity of these channels is under the influence of sex-steroid. Steroids influence the kinetic properties of both anion and cation channels (Ashvani, *et al.*, 2000) and the expression of these epithelial channel are regulated by estrogenic hormone (Rochwerger & Buchwald, 1993). The expression of the different protein channel along with CA will be discussed in the endometrium.

### *2.2.7.1 Carbonic Anhydrase (CA)*

CA is a metalloenzyme which belongs to α CA family, that contain zinc ion and enclosed by histidine residues and one water molecule (Karhumaa, 2002). CA is involved in $H^+$ and $HCO_3^-$ production (Sly & Hu, 1995) and transportation (Wu, *et al.*, 1997) via reversible hydration of $CO_2$. This can influence the environment's acidity (Barnett, *et al.*, 2008), and facilitate $CO_2$ transport across the membrane (Hynninen, *et al.*, 2004). CA is the main enzyme involved in the transport and metabolic process at the cellular level (Henry, 1996). Sixteen different isoenzymes for CA have been identified in mammals (Liao, *et al.*, 2009). These are participated in a wide range of physiological events such as respiration, acid-base balance, ion transport, bone absorption, renal acidification, gluconeogenesis, and ureagenesis, formation of aqueous humor, cerebrospinal fluid, saliva, and gastric acid (Henry, 1996).

CA is involved in different reproductive process including capacitation and sperm maturation via the $HCO_3^-$ secretion pathway (Boatman, 1997; Hu & Spencer, 2005). Furthermore, CA contributes to development of neonatal mouse uterus gland (Hu & Spencer, 2005). CA may be involved in the changes of uterine fluid pH through bicarbonate production (Hynninen, *et al.*, 2004). Participation of CA in uterine $HCO_3^-$ production has been reported, whereby application of acetazolamide (ACTZ, a CA inhibitor), to the basolateral side of cultured mouse endometrial reduced current in normal and $Cl^-$ free buffer. However $HCO_3^-$ free buffer had no effect, indicating low contribution of CA in $HCO_3^-$ production (Fong, *et al.*, 1998b)

Furthermore, application of ACTZ reduced spermatozoa volume in fowl and decreased rate of testicular fluid secretion. It also inhibited the acidification of epididymal fluid (Breton, *et al.*, 1996). In rabbit, ACTZ leads to lower numbers of implantation. It further terminates pregnancy after direct administration into the uterus (Pincus & Bialy, 1963). However, Chiang, *et al* (2004) reported that the CA inhibitor sulphonamide

when administered on day 2.5 of pregnancy has a small effect on implantation in mice. He suggested that cytosolic CA is a negative regulator of decidualized uterine tissue stability that is involved in acid-base balance in the uterus and creates a microenvironment conducive to cell growth and spread.

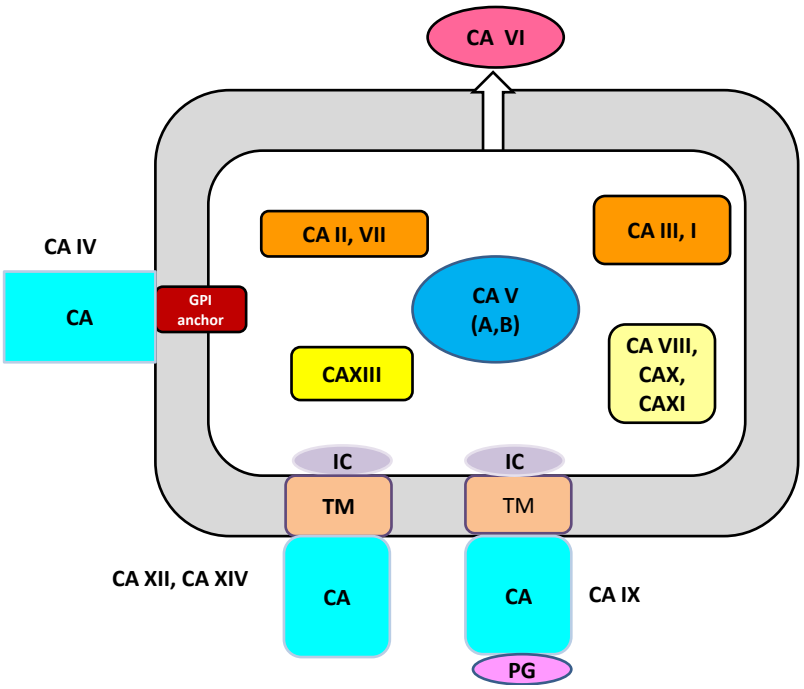



**Figure 2. 2** Molecular structures and sub cellular localization of CA isoenzyme. CA isoenzyme are intra cellular such as CAII and VII ( high activity enzyme), CA XIII (medium activity) , CA I and III (low activity) , CA VIII, X and XI (no activity) and CA V (mitocondrial ) or extra cellular such as CA IX and XII ( high activity), CA IV and XIV (medium activity). CA: carbonic anhydrase, GPI: glycosyl phosphatidyl inositol, PG: proteoglycan like domain, TM: transmembrane domain, IC: intracytoplasmic tail. (Adapted from Nishimori & Onishi, 2001).

CA expression and activity is under the influence of ovarian hormones (Lutwak-Mann, 1955) and its expression varies in different species. Unique isoenzymes of CA are induced by P in human or rabbit uterus (Hodgen & Falk, 1971), and in some species such as guinea pig estrus stimulate CA activity (Lutwak-Mann, 1955). In lactating cows CA increased between estrus and day nine of the oestrous cycle (Elrod, *et al.*, 1993) . Down regulation of cytosolic CA (CAII) during implantation that can lead to acidification and enhance cell proliferation has been observed. In view of this, Chiang, *et al* (2004) showed that in mice abortion induced by substance P (neuropeptide), resulted in significant expression of cytosolic CA and decreased the number of

embryos. In rat prostate and seminal vesicle, CA activity is regulated by androgen (Pincus & Bialy, 1963). However in sheep CA is free from ovarian hormone influences (Lutwak-Mann, 1955).

CA has been detected in ovarian epithelium, granulosa cell of maturating follicle, and oviduct smooth muscle (Ge & Spicer, 1988). Endometrial CA activity has been detected by histochemical studies 30 years ago and later different isozyme have been identified (Karhumaa, *et al.*, 2000). For example, expression of CAXII in human basolateral membrane of endometrium (Karhumaa, *et al.*, 2000), CAXIII in mouse endometrium has been detected in deeper endometrial glands whereas CAIX signal is very weak (Hynninen, *et al.*, 2004). CAII has been found in human endometrial capillaries, whereby this high activity isoenzyme is abundantly expressed in the cytoplasm and epithelial cell membrane of the mouse endometrium (Hynninen, *et al.*, 2004). Since the presence of CAII in mice endometrium, and CAXII in human endometrium has been reported; in this study the expression of these isoenzymes under the influence of ovarian hormone was evaluated.

*2.2.7.1.a CAII*

CAII is located on chromosome 8q22 in human, and generates a protein with 29kDa (Venta, *et al.*, 1991). CAII is a very active isozyme with the maximum turnover rate for $CO_2$ hydration. It can be detected in almost all cells such as osteoclasts in bone, oligodendrocytes in brain, epithelium of the choroid plexus (brain) and the ciliary body (eye), lens, miller cells in retina, liver (mainly perivenous hepatocytes), kidney acinar cells in salivary glands, pancreatic duct cells, gastric parietal cells, endometrium of the uterus, endothelial cells, epithelial cells of seminal vesicle and ductus deferens, spermatozoa, erythrocytes, and platelets. It has also been reported in neutrophils, type II epithelial cells of lung, endothelial cells and epithelial cells of duodenum, intestine, and colon, and zona glomerulosa cells of the adrenal (Sly & Hu, 1995).

The role of CA II varies in different organs. In gastric parietal cells and renal tubular, it is involved in fluid acidification. However, in pancreatic duct, choroid plexus and salivary gland, $HCO_3^-$ secretion is attributed to CAII activity. Due to its role in $H^+$ and $HCO_3^-$ absorption and secretion, CA promotes water and NaCl absorption in the large intestine, gallbladder, in erythrocytes, lungs and kidneys (Sly & Hu, 1995).

*2.2.7.1.b CAXII*

CAXII is mapped to chromosome 15q22 in human and produces type 1 membrane protein which is 43-44kDa in size (Türeci, *et al.*, 1998). CAXII is an active isoenzyme and its mRNA is expressed in efferent ducts, prostate gland, testis, ovary, pancreas lungs and brain (Ivanov, *et al.*, 2001). It is over expressed in some cancer tissues and cell lines (Ivanov, *et al.*, 2001; Parkkila, *et al.*, 2000). However CAXII protein has only been detected in endometrium, colon and kidney (Karhumaa, 2002; Parkkila, *et al.*, 2000). Liao, *et al* (2009) has reported the expression of CAXII occurs in cells derived from the mesonephric duct or cells that involved in secretion and proton pumping-pH regulation. CAXII is probably related to water and $HCO_3^-$ absorption in different epithelial cells such as in the efferent duct and kidney. This has been confirmed by simultaneous expression of CAXII and AQP in these cells (Karhumaa, *et al.*, 2001b; Parkkila, *et al.*, 2000). Moreover, inhibition of basolateral CA in kidney proximal tubule decreases the $HCO_3^-$ and fluid absorption. The presence of CAXII in the endometrium is related to CAXII activity in a pH dependent event that is involved in fertilization (Chiang, *et al.*, 2004; Karhumaa, *et al.*, 2000). It is also responsible for acidification of luminal epididymal fluid in apical mitochondria-rich cells (AMRC) and in tumours (Ivanov, *et al.*, 1998; Karhumaa, 2002). Expression of CAXII in the male reproductive system lead to morphological changes in ER α in knockout mice, whereby this receptor is essential for fluid reabsorption in the efferent duct (Zhou, *et al.*, 2001). In human breast tumour, and in mouse uterus, expression of CAXII is under the stimulatory effect

of E (Barnett, *et al.*, 2008). Involvement of CAXII in cell proliferation and oncogenesis has been reported (Breton, 2001).

*2.2.7.1.c CA deficiency*

Among CA family member only deficiency in CA II leads to known disease, including osteopetrosis, renal tubular acidosis, and cerebral acidification (Sly & Hu, 1995).

***2.2.7.2 Bicarbonate transporter***

The uterine $HCO_3^-$ content is higher than plasma indicating active $HCO_3^-$ transport across the endometrium (Wang, *et al*., 2003b). The $HCO_3^-$ transporter contributes in regulation of pHi, cell volume changes along with acid-base equivalent and $CO_2$ flux across the cell membrane (Boron & Boulpaep, 1989). The following discussion continues on the $HCO_3^-$ transporter that may involve in the regulation of uterine $HCO_3^-$ content.

The bicarbonate transporter super family includes NBC, $Cl^-$/ $HCO_3^-$ exchanger, and $Na^+$ driven $Cl^-$/ $HCO_3^-$exchanger (Figure 2.3) (Kurtz, *et al*., 2004). There are a few differences between NBC and other transporters: NBC is not $Cl^-$ dependent, and the presence of $HCO_3^-$ stimulates NBC (Abuladze, *et al.*, 1998). However all of mentioned transporter were inhibited by stilbenes such as DIDS (Boron & Boulpaep, 1989). CFTR is also considered a $HCO_3^-$ transporter that is activated by cAMP in different epithelial cells (Choi, *et al.*, 2001).

Acid- base transporter can be divided into" acid loader", which brings net acid into the cell or "acid extruder" that remove net acid from the cell (Roussa, 2011) (Figure 2.4).

Acid extruders include NHEs from the SLC9 family, proton channels, and proton pumps. In addition $Na^+$-coupled $HCO_3^-$ transporters (Figure 2.3) and the electrogenic NBCe1/-e2 (with a stoichiometry of $1Na^+$:$2HCO_3^-$) from SLC4 family are acid extruders. Acid loaders consist of NBCe1/e2 (with a stoichiometry of $1Na^+$:$3HCO_3^-$)

and the anion exchangers from the SLC4 family, SLC26 family and $HCO_3^-$ permeable channels like CFTR.

Nevertheless, the direction of ion flux via a transporter might change in different tissues/cells depending on the local electrochemical driving force existing across the plasma membrane. pH homeostasis relies on balanced activities of the acid extruders and acid loaders.

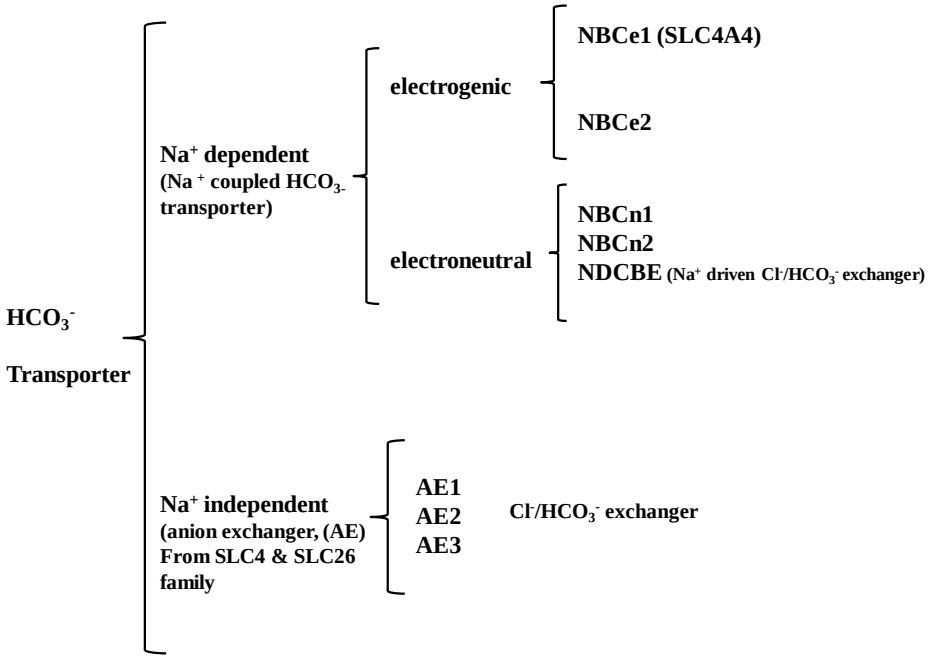



**Figure 2. 3** Different bicarbonate transporter.

### *2.2.7.3 Cystic fibrosis transmembrane conductance regulator (CFTR)*

CFTR is a transmembrane protein (Figure 2.5) and member of the ATP binding cassette transporter family (Chan, *et al.*, 2006) located on the chromosome 7 with 27 exon and 250kbp (Bargon, *et al.*, 1992).

CFTR is mostly expressed in the epithelial cell, although it is found in some non-epithelial tissue like cardiac myocytes (Bargon, *et al.*, 1992; Hwang & Sheppard, 1999).

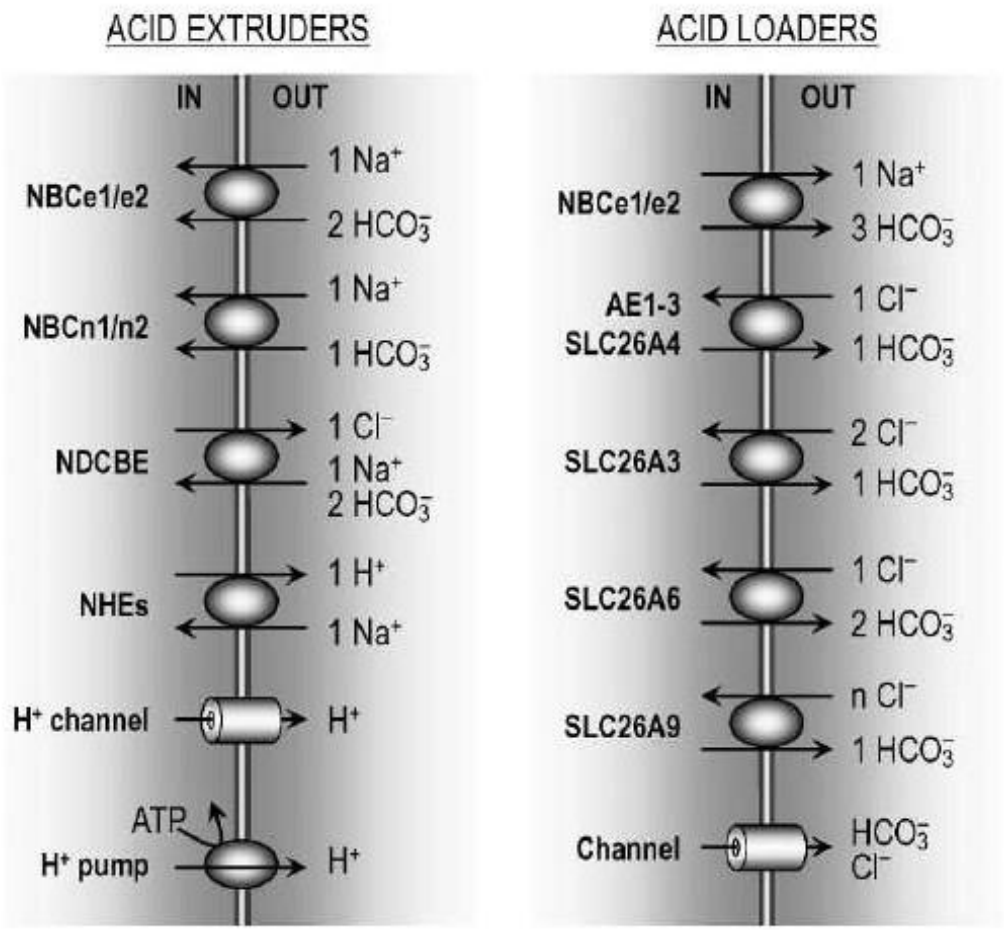



**Figure 2. 4** Ion transport modes of acid-base transporters. IN, intracellular; OUT, extracellular.
**Source**: (Liu, *et al.*, 2012)

CFTR is distributed in wide variety of systems as well as reproductive organs (Fiedler, *et al.*, 1992; Snouwaert, *et al.*, 1992). In the female, CFTR expression in the luminal and glandular epithelium of the uterus, ovary, oviduct, cervix and vagina has been reported where its regulation is steroid dependent (Trezise, *et al.*, 1993). It has been reported that the expression of CFTR in uterine epithelial cell line (UTT 1.16), is E dependent, and CFTR mRNA has been observed in the presence of E. Furthermore E up regulated CFTR expression in immature and ovariectomized mature rat (Rochwerger & Buchwald, 1993; Rochwerger, *et al.*, 1994). Chan, *et al* (2002) has reported maximal expression of CFTR occurs at proestrus in the cervical and vaginal epithelia although in uterine epithelium CFTR mRNA was only detected at early estrus. In addition there is large amount of CFTR in ovary and oviduct throughout the oestrous cycle. CFTR

however is down-regulated by P in mouse endometrium (Tsang, *et al.*, 2001). In fetal rat lung, androgen increases while E decrease CFTR functional activity (Sweezey, *et al.*, 1997)

CFTR responds to increased levels of intracellular cyclic AMP (cAMP) by elevating secretion of $Cl^-$ from cells (Bargon, *et al.*, 1992), thus regulating the rate of transepithelial salt and water transport (Hwang & Sheppard, 1999). PKA activates CFTR via phosphorylation (Zhang, *et al.*, 2000). However CFTR is also implicated in cAMP dependent $HCO_3^-$ secretion in the apical face of the endometrial epithelium in mice. Furthermore, CFTR participates in $HCO_3^-$ conductance in airway, intestine and pancreas (Wang, *et al.*, 2003a).

Different CFTR regulators including cAMP, tumour necrosis factor, phorbol ester, divalent cations ionofor and E have been reported (Rochwerger, *et al.*, 1994).

Increased activity of the CFTR $Cl^-$ channel, may be involved in different diseases such as poly cystic kidney diseases and secretory diarrhoea (Hwang & Sheppard, 1999). Mutations in the gene encoding CFTR lead to lethal autosomal recessive disorder cystic fibrosis (CF). CF patients are affected by chronic obstructive lung disease, pancreatic insufficiency, intestinal malabsorption and obstruction, and have elevated levels of sweat electrolytes, due to atypical regulation of electrolyte transport in affected organs. It has documented that in CF patients, reproductive organs are affected in both genders. In the male anatomical abnormality in the epididym, in the female endocervicitis, uterine polyp, thick cervical mucus, alteration in cervical mucus content and failure in sperm capacitation has been reported without any anatomical deficiency (Bargon, *et al.*, 1992; Rochwerger, *et al.*, 1994; Wang, *et al.*, 2003a) .

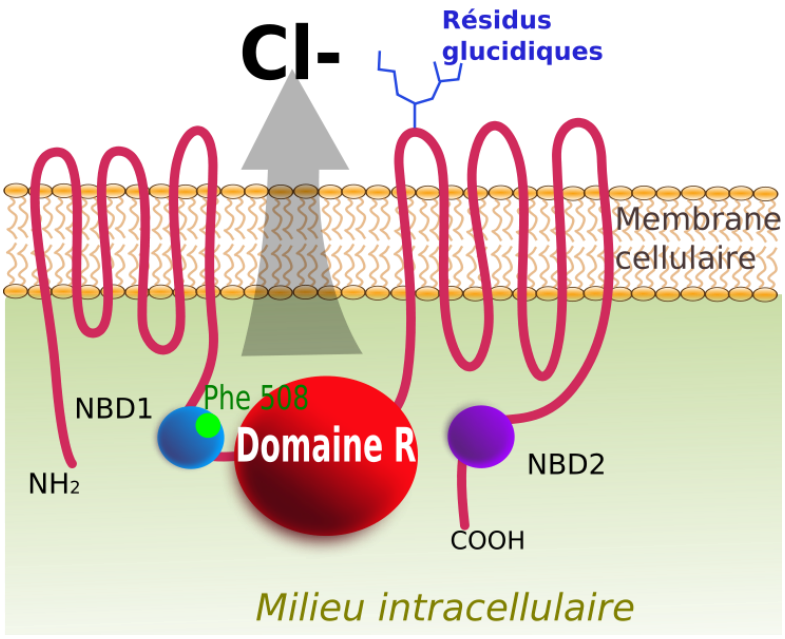



**Figure 2. 5** CFTR molecule contain two non identical halves, each part has six transmembrane spanning domain, that connected to nucleotide binding domain (NBD) in each side, NBD1 and NBD2 attached to the regulatory domain (R)(Zhang, *et al.*, 2000). Some CFTR stimulator , interact with NBD that control channel gating, whereas CFTR inhibitors bind to channel pore and prevent Cl- permeation.

**Source:** (Toony, 2008)

### *2.2.7.4 Chloride/bicarbonate exchanger*

Three main members of $Cl^-$/ $HCO_3^-$ exchangers, group of AE: AE1, AE2 and AE3, are encoded by three different genes in mammals. The protein is homologous to band 3 in erythrocyte which functions as a $Cl^-/HCO_3^-$exchanger. The $Cl^-/HCO_3^-$ exchanger participates in various physiological process including regulation of pHi, cell volume and acid/base transport in a wide range of epithelial cells (Jensen, *et al.*, 1999b). While three families encode different polypeptides in different cells and their exchanger activity and probably their regulatory mechanism vary in distinct cells (Zhao & Baltz, 1996). They all mediate $Cl^-$/ $HCO_3^-$ exchanger and their anion specificity is similar. The $Cl^-$/ $HCO_3^-$ exchanger might respond to different physiologic stimulus and contributes in different physiologic functions. As an example it takes part in net acid or net base secretion in type A or type B intercalated cells respectively. In the medullary thick ascending limb ok kidney $Cl^-$/ $HCO_3^-$ exchanger is involved in volume regulation (Jensen, *et al.*, 1999b).

The AE family contains two main types of exchangers, $Na^+$ dependent and $Na^+$ independent. $Na^+$ dependent exchanger known as acid extruder (Figure 2.4), relies on the presence of $Na^+$, the $Na^+$ gradient drives $HCO_3^-$ inside the cell and increase pHi. The second type is $Na^+$-independent exchanger, which known as band 3 in red blood cells. It is involved in $Cl^-$ shift and mediate a $HCO_3^-$ efflux, thereby reduce the intracellular pH (Boron & Boulpaep, 1989)

AE has been detected in the luminal membrane of secretory epithelia (Dorwart, *et al.*, 2008), during preimplantation stages in mice embryo, and is involved in pHi and cell volume regulation (Zhao & Baltz, 1996)

The AE member of the uterus is SLC26A6, and it is located in the luminal membrane of secretory epithelia. It plays a role in $Cl^-$ absorption and $HCO_3^-$ secretion and function as a $Cl^-/HCO_3^-$ exchanger with a stoichiometry $1Cl^-/2HCO_3^-$. This role is crucial in production of high $HCO_3^-$ fluid in varies epithelia such as pancreatic duct (Dorwart, *et al.*, 2008; Melvin, *et al.*, 1999).

There are different regulatory mechanisms for the SLC26 family, including transcriptional, protein trafficking and post translational modification (Lee, *et al.*, 2003). Additionally PGE2 stimulate SLC26A6 activity (Dorwart, *et al.*, 2008).

There is an association between CFTR and $Cl^-/HCO_3^-$ exchanger activity in the regulation of fluid secretion in pancreatic duct, in which SLC26A6 inhibit activity of CFTR in resting state and in stimulated state it raises the $Cl^-$ channel activity of CFTR (Dorwart, *et al.*, 2008). However there is lack of information about the expression of SLC26A6 under the influence of ovarian hormone, thus in this study it will evaluated.

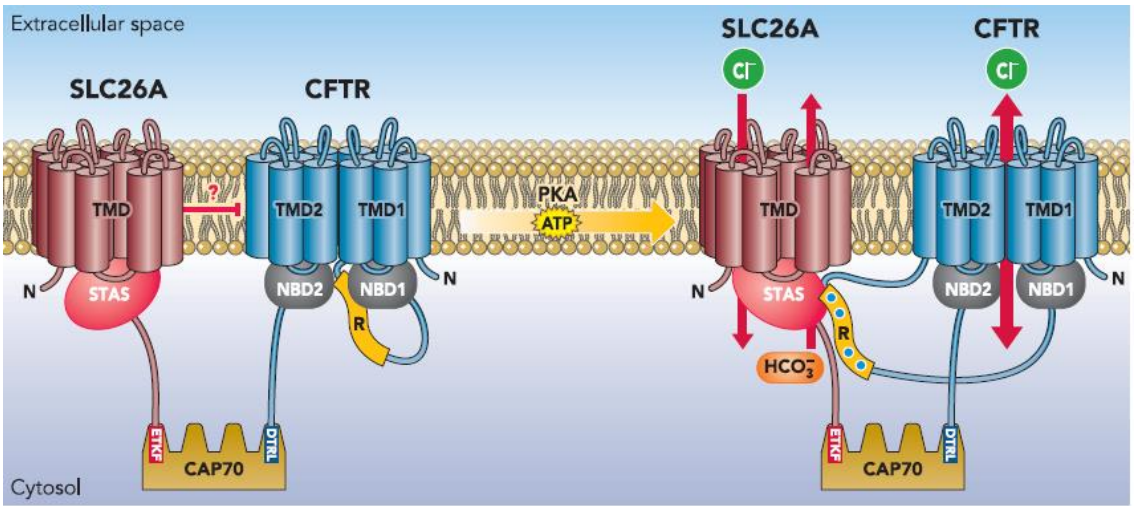



**Figure 2. 6** Regulatory interaction between SLC26 transporters and CFTR regulates resting and stimulated fluid and electrolytes secretion.A PDZ domain-containing scaffolding protein assembles luminal membrane complexes of CFTR and the SLC26 transporters.To avoid unnecessary secretion in the resting state, the nonphosphorylated R domain interacts with NBD1 to prevent its interaction with NBD2 and activation of CFTR Cl– channel activity . This interaction and inhibition of the spontaneous activity of CFTR is stabilized by SLC26A6 , by as yet unknown mechanism, to inhibit fluid and electrolyte secretion in the resting state. Activation of PKA phosphorylates the R domain to alter its binding to NBD1 and at the same time enhances its binding to the STAS domain . This results in the mutual activation of CFTR and the SLC26 transporters and activation of fluid and electrolyte secretion . This form of regulatory interaction has been demonstrated so far for slc26a3, SLC26A4, and slc26a6 but is likely to operate with other SLC26 . **Source**: (Dorwart, *et al.*, 2008)

### *2.2.7.5 Sodium- bicarbonate transporter*

NBC is involved in concomitant $Na^+$ and $HCO_3^-$ movement across the cell membrane of many cells (Boron & Boulpaep, 1989) and the direction of ions flux is cell dependent (Gross, *et al.*, 2003). This coupled transport contributes to $HCO_3^-$ secretion/absorption and pHi regulation (Jensen, *et al.*, 1999a) and extracellular acid–base regulation (Kurtz, *et al.*, 2004). The stoichiometry of ions flux varies between cells and is $3HCO_3^-/1Na^+$ ($HCO_3^-$ efflux) or 2 $HCO_3^-/1Na^+$ ($HCO_3^-$ influx) in kidney and pancreatic duct respectively (Gross, *et al.*, 2001). The stoichiometry of NBC is regulated by cell specific factor and PKA is able to switch between these two modes (Gross, *et al.*, 2003). Two well-known NBC1 member are located in pancreas (pNBC) and kidney (kNBC) (Abuladze, *et al.*, 1998) and are from the SLC4A4. There are 994 amino acid similarities in their C-terminal, while they differ in N-terminal. kNBC is specific to

kidney but pNBC has been detected in other organ such as brain, spinal cord, colon, kidney, liver, prostate, stomach and thyroid (Abuladze, *et al.*, 1998). Another NBC1 variant that differs in the COOH terminal has been identified in rat brain (Roussa, 2011). In kidney NBC is located on the basolateral membrane of proximal tubule and is involved in electrogenic $HCO_3^-$ flux (Abuladze, *et al.*, 1998; Yoshitomi, *et al.*, 1985). In pancreas basolaterally located NBC contributes to 75% of $HCO_3^-$ uptake during apical $HCO_3^-$ secretion, whereby the $Cl^-$/base exchanger and CFTR secrete $HCO_3^-$ into the lumen and provide $HCO_3^-$ rich fluid (Abuladze, *et al.*, 1998; Ishiguro, *et al.*, 1996a; Ishiguro, *et al.*, 1996b). The basolateral application of H2DIDS (an NBC inhibitor), lead to 80% reduction in $HCO_3^-$ secretion, indicating involvement of NBC in $HCO_3^-$ uptake from the basolateral side of mouse endometrium (Fong, *et al.*, 1998b; Wang, *et al.*, 2002).

NBC activity is dependent on internal pH sensitive site of the protein that inhibits its activity in alkaline pH and affects $Na^+$ and $HCO_3^-$ flux (Gross & Hopfer, 1999; Soleimani, *et al.*, 1991).

NBC activity is associated with the CAII and the $Cl^-/HCO_3^-$ exchanger. There is close interaction between C-terminal of AE1 and amino-terminal of CAII. CAII stimulates AE activity, thus inhibition of CAII by ACTZ reduces AE function. NBC1/AE1 share the homologues region at C-terminal and the C-terminal of kNBC1 has interaction with CAII (Gross, *et al.*, 2001, 2002, &2003).

Phosphorylation of kNBC or pNBC following PKA activation lead to dissociation of CAII from the transporter and decreases the effect of CAII inhibition (Gross, *et al.*, 2003; Sterling, *et al.*, 2001a, 2001b; Vince & Reithmeier, 1998), and thus can influence both NBC and AE.

NBC expression and functional activity has been reported to be regulated by multiple factors including hormones and paracrine agents. Parathyroid hormone has been

reported to induce basolateral membrane NBCe1 expression, thus increasing intestinal $HCO_3^-$ secretion, particularly in the ileum (Charoenphandhu, *et al.*, 2011). Secretin has been reported to increase basolateral $HCO_3^-$ uptake in the pancreatic duct (Futakuchi, *et al.*, 2009). Angiotensin II has been reported to inhibit the electrogenic NBC activities in cardiac myocyte (De Giusti, *et al.*, 2010). As the female reproductive tissues usually bear E and P receptors (ER & PR) and are sensitive to the influence of sex-steroids (Samartzis, *et al.*, 2012), uterine NBC expression and activities would probably be influence by these two hormones. In view of this, this study was conducted to investigate the changes in the expression as well as functional activity of this protein SLC4A4 under the influence of sex-steroid hormones and at different stages of the oestrous cycle in a rodent model.

#### *2.2.7.6 Sodium-Hydrogen exchanger*

NHE isoforms are integrated plasma membrane protein that exchange intracellular $H^+$ with external $Na^+$ with stoichiometry of $1H^+$: $1Na^+$, according their concentration gradient and in reversible fashion. They belong to the solute carrier family9 of exchanger, (SLC9) (Chavez, *et al.*, 2011; Yun, *et al.*, 1995b). Mammalian NHE consists 9 member (NHE1-9), and are present in almost all living cells (Dunham, *et al.*, 2004; Orlowski & Grinstein, 2004). NHE is involved in cell volume changes, pHi regulation and acid-base balance (Praetorius, *et al.*, 2000; Wang, *et al.*, 1993). Its activity facilitates cellular adhesion, migration and proliferation. NHE is one of the major non-nutritive $Na^+$ absorptive pathway in intestine and rat kidney (Praetorius, *et al.*, 2000).

NHE family genes encode proteins with 25-70% identity, although they share secondary structure that consist 12 conserved membrane spanning region at the amino terminal and divergent cytoplasmic carboxy terminal segment (Orlowski & Grinstein, 2004). The expression pattern and membrane localization vary among different isoforms. Furthermore, they differ in kinetic properties and sensitivity to inhibitors, and probably

they vary in hormonal regulation, mechanical stimulation and responses to growth factor and protein kinase (Orlowski & Grinstein, 2004; Yun, *et al.*, 1995a).

Wide variety of molecular signal are able to effect the NHE activity such as neurotransmitter, growth factor, peptide hormone, phorbel ester, cAMP, chemotactic factors, glucocorticoid and thyroid hormone (Kandasamy & Orlowski, 1996)

NHE exchange extracellular $Na^+$ with intracellular $H^+$, leads to acid extrusion that accumulates during cell metabolism; hence NHE along with $HCO_3^-$ transporter contributes to the regulation of pHi (Orlowski & Grinstein, 2004).

NHE probably is involved in $HCO_3^-$ absorption from the epithelial cell, for example, 15% of $HCO_3^-$ absorption in thick ascending Henle's loop is dependent to luminal NHE (Good, 1985). Furthermore in epididymis NHE2 and NHE3 are contributed in $HCO_3^-$ transport that resulted in luminal acidification (Bagnis, *et al.*, 2001). In salivary gland NHE involved in both fluid absorption and secretion activity (Martinez & Cassity, 1985). The presence of three NHE isoform in uterus has been reported, NHE1,2 and 4(Wang, *et al.*, 2003b).

*2.2.7.6.a NHE1*

NHE1 widely present in almost all mammalian cell such as intestine, kidney, red blood cell, brain and male reproductive tract (Chew, *et al.*, 2000; Douglas, *et al.*, 2001; Dunham, *et al.*, 2004). It has a house keeping role and is involved in the pHi and cell volume homeostasis by extruding $H^+$ out and $Na^+$ into the cell (Baumgartner, *et al.*, 2004; Bookstein, *et al.*, 1994; Praetorius, *et al.*, 2000). NHE1 consists of 500 amino acid transport domain that extend into the cytoplasm and contain 12 membrane spanning domain (Wang, *et al.*, 2003b). NHE1 activity is regulated by pHi, and intracellular acidosis increase NHE1 activity through the interaction between $H^+$ and pHi sensor of NHE (Haworth, *et al.*, 2003). NHE1 in parallel with $Cl^-/HCO_3^-$ exchanger facilitated NaCl and water flux, thus they involved in cell volume regulation in red

blood cell (Dunham, *et al.*, 2004). The interaction of NHE1 with $Cl^-/HCO_3^-$ exchanger and NBC maintains cytoplasmic pH and acid-base balance. NHE1 participates in fluid absorption in male reproductive tract, in which amiloride reduced fluid absorption in the epididymis (Karhumaa, 2002). NHE1 is involved in cell morphology, adhesion and migration, probably via remodeling the cortical actin cytoskeleton (Orlowski & Grinstein, 2004). NHE1 contain a relatively conserved N-terminal, transmembrane ion translocation domain and a cytoplasmic regulatory C-terminal domain that serve as a target for a different regulator factor (Orlowski & Grinstein, 2004).

*2.2.7.6.b NHE2*

NHE2 has been detected in intestine, gastrointestinal tract, lungs and skeletal muscles (Bookstein, *et al.*, 1997; Chambrey, *et al.*, 1998; Hoogerwerf, *et al.*, 1996; Wang, *et al.*, 1993). $HCO_3^-$ reabsorption in the distal convoluted tubule is regulated by $H^+$ secretion via NHE2 (Malakooti, *et al.*, 1999; Orlowski & Grinstein, 2004; Orlowski, *et al.*, 1992). NHE2 is involved in net $Na^+$ and $H^+$ transepithelial transport in several absorptive epithelia (Bagnis, *et al.*, 2001). It has 42% similarity to NHE1 (55% in N- terminal transmembrane domain and 25% in C-terminal cytoplasmic domain), while NHE2 and NHE3 have 36% in common in which 48% similarity is in transmembrane domain and 21% similarity for cytoplasmic region. Similarity of NHE2 with NHE4 is approximately 57% (63% for transmembrane and 46% cytoplasmic region).

*2.2.7.6.c NHE4*

In the kidney NHE4 is located on the basolateral side of renal epithelia, where its function may overlap with NHE1 (Orlowski & Grinstein, 2004). It is also detected in cortical collecting duct, macula densa (Peti-Peterd, *et al.*, 2000) and intestine (Bagnis, *et al.*, 2001). NHE4 has been reported to be active under hypertonic condition and thus involved in cell volume regulation (Bagnis, *et al.*, 2001).

NHE4 basolateral location is not affected by acids –base disturbances (Oehlke, *et al.*, 2006). It has been reported that NHE4 is not active under most physiological condition (Bookstein, *et al.*, 1997)

## 2.3 Interaction between CA and other transporters in different epithelial cells

It has been suggested that CAII and CAIV (an active, membrane enzyme), form an active metabolon with AE isoforms and NHE1 (Breton, 2001; Hynninen, *et al.*, 2004). In epididym CAIV involved in $HCO_3^-$ reabsorption while CAII supplying $H^+$ for secretion mediated by different ion transporters (Kaunisto, *et al.*, 1995). CA II along with PP contributes to epididymal luminal acidification which is dependent on the $HCO_3^-$ (Breton, *et al.*, 1998). In addition to CA, epididymal luminal acidification, is $Na^+$ independent in proximal vas deferens, while in cauda epididym is $Na^+$ dependent that indicating that NHE has a role (Au & Wong, 1980; Jensen, *et al.*, 1999a).

In pancreatic duct, CAs contributes in self-defense mechanism against luminal acidification. Under acidic condition, CAs catalyze $H^+ + HCO_3^- \rightarrow CO_2 + H_2O$, thus removing extra acid from the lumen while $CO_2$ passing through the membrane and enter the blood or renters the $HCO_3^-$ cycle (Nishimori & Onishi, 2001). $CO_2$ is only responsible for 50% of $HCO_3^-$ in pancreatic juice, and the high $HCO_3^-$ level is accumulated by $Cl^-/ HCO_3^-$exchanger along with CFTR and NBCe1 activity in the apical membrane and $HCO_3^-$ uptake in the basolateral membrane through NBC or $Na^+$ dependent $Cl^-/ HCO_3^-$exchanger (Gross, *et al.*, 2001; Gross, *et al.*, 2002; Ishiguro, *et al.*, 1996a). Furthermore in pancreas, CFTR act as a key regulator of different protein channels such as NBC, SLC26A6 and NHE3 (Steward & Ishiguro, 2009).

In colon, CA contributes in electroneutral NaCl absorption via synchronous operation of apical NHE and $Cl^-$/ $HCO_3^-$ exchanger. Thus by supplying $HCO_3^-$ to $Cl^-$/ $HCO_3^-$ exchanger, CA involved in alkalization of luminal content (Halmi, *et al.*, 2004).

Net $HCO_3^-$ absorption in kidney results from $H^+$ secretion into the lumen by NHE3 and PP. Then in the lumen, $H^+$ combines with filtered $HCO_3^-$ by CAIV and produce $CO_2$. $CO_2$ across the membrane and in the cytoplasm converted to $HCO_3^-$ via CAII, which exit from the cell by basolateral NBC (Seki, *et al.*, 1996).

It has been reported that in non-pigmented ciliary epithelium in pig, CAII and CA IV in coordination with AE2 are involved in aqueous humour secretion through affecting $HCO_3^-$ transport into the eye (Shahidullah, *et al.*, 2009).

In salivary gland, NHE activity coupled with $Cl^-$/ $HCO_3^-$ exchanger is associated with pH regulation. In acinar cells NHE and CAII activity might enhance the $Cl^-$/ $HCO_3^-$ exchanger activity via accumulation of $HCO_3^-$. Application of acetazolamide reduces the $Cl^-$/ $HCO_3^-$ exchanger activity (Nguyen, *et al.*, 2004).

Taken together this indicates that close interaction exists in among different transporter that influence net $H^+$ and $HCO_3^-$ transport.

## Chapter 3

# Methodology

# 3- Methodology

## 3.1 Chemicals and consumables

4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), acetazolamide, N-ethyl-N-isopropyl amiloride (EIPA), 17 beta-oestradiol, progesterone, glibenclamide, acetazolamide and ammonium persulfate (Sigma, St. Louis, USA).

Pro-Prep protein extraction solution (Intron Biotechnology, Korea). Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, USA). βeta actin antibody (Abcam, UK). 4CN optic substrate kit and Polyvinylidene fluoride membrane (PVDF) (Bio Rad, Alfred Nobel Drive Hercules, CA, USA).

Bovine serum albumin (Innovative, Peary Court Novi, Michigan, USA). Spectra multicolor broad range (Fermentas, Rockford, USA). Paraformaldehyde, TEMED (Merck, Germany). ABC staining kit and all primary and secondary antibodies (Santa Cruz, Delaware Avenue,CA, USA), Polylysine coated slide (Menzal, Germany). DPX mounting medium (Sigma, St. Louis, USA).

Micro Amp fast 8 tube strip, Micro Amp Optical cap strip. RNAlater, Taq Man RNA to $C_T$ 1step, and RNA assays (Applied Biosystems,Lincoln Centre Drive, Foster City, USA). Ribo pure kit, RNase away and RNAlater® (Ambion, Lincoln Centre Drive, Foster City, USA).

## 3.2 Sterilization

Solutions, plastic and glass disposables were sterilized by autoclaving for 20 min at 1.05 kg/cm2 (15 psi) on liquid cycle. Distilled water ($dH_2O$) was used for analytical purposes throughout the study. Nuclease-free $H_2O$ was used for studies with RNA.

## 3.3 Approval by Animal Ethic Committee

Animal studies were approved by Medicine Animal Care and Use Committee (ACUC) University of Malaya, the Ethic number is 14/9/2009/FIS/NS. Wistar Kyoto (WKY), female rats (Rattus norvegicus), approximately 2 months of age and 180-230 g weight were obtained from animal house of University of Malaya. Rats were caged in groups of 4 in polycarbonate cage, 19" X 10" X 6" high with bedding in a clean and well ventilated animal Laboratory in animal house of the Department of Physiology, Faculty of Medicine, University of Malaya. Rats were maintained with a standard cycle of 12 hours light (light is on at 7 a.m), and 12 hours dark, room temperature kept at ± 25°C, and humidity of 30-70%. Animals were provided with soy-free diet (Gold Coin Pellet, from Altromin, Germany) and tap water *ad libitum*.

## 3.4 Study samples

Rats were divided into different groups (table 3.1). In-vivo uterine perfusions were conducted in all groups to analyze the changes in the pH, secretion rate, $Na^+$, $Cl^-$ and $HCO_3^-$ content of the uterine fluid. Following perfusions, the animals were humanely sacrificed and the uterine horns were collected for Western blotting, immunohistochemistry (IHC) and quantitative real time PCR (qPCR).

**Table 3. 1** Different experimental groups that have been used in this study.

| | | Perfusion without inhibitor<br><br>Western blot, IHC, qPCR | Perfusion in the presence of different inhibitors |
|---|---|---|---|
| Female WKY Rats | Ovariectomized rats | 1- Control (3 days peanut oil) | I. Control (perfusion with glibenclamide)<br>II. Control (perfusion with DIDS)<br>III. Control (perfusion with ACTZ)<br>IV. Control (perfusion with EIPA) |
| | | 2- 0.2µg estrogen,3 days (0.2E) | I. 0.2E (perfusion with glibenclamide)<br>II. 0.2E (perfusion with DIDS)<br>III. 0.2E (perfusion with ACTZ)<br>IV. 0.2E (perfusion with EIPA) |
| | | 3- 4 mg progesterone (3 days) (P) | I. P (perfusion with glibenclamide)<br>II. P (perfusion with DIDS)<br>III. P (perfusion with ACTZ)<br>IV. P (perfusion with EIPA) |
| | | 4- 0.2µg estrogen + 4mg progesterone, each for 3 days, (E+P)* | I. E+P (perfusion with glibenclamide)<br>II. E+P (perfusion with DIDS)<br>III. E+P (perfusion with ACTZ)<br>IV. E+P (perfusion with EIPA) |
| | | 5- 2µg estrogen (3 days)<br>6- 20µg estrogen (3 days)<br>7- 50µg estrogen (3 days) | |
| | Intact Rats | 8- Proestrus (Ps) | I. Ps (perfusion with glibenclamide)<br>II. Ps (perfusion with DIDS)<br>III. Ps (perfusion with ACTZ)<br>IV. Ps (perfusion with EIPA) |
| | | 9- Estrus (Es) | I. Es (perfusion with glibenclamide)<br>II. Es (perfusion with DIDS)<br>III. Es (perfusion with ACTZ)<br>IV. Es (perfusion with EIPA) |
| | | 10- Metestrus (Ms) | I. Ms (perfusion with glibenclamide)<br>II. Ms (perfusion with DIDS)<br>III. Ms (perfusion with ACTZ)<br>IV. Ms (perfusion with EIPA) |
| | | 11- Diestrus (Ds) | I. Ds (perfusion with glibenclamide)<br>II. Ds (perfusion with DIDS)<br>III. Ds (perfusion with ACTZ)<br>IV. Ds (perfusion with EIPA) |

Perfusion was performed with and without inhibitors. In groups 5-7, in which the rats were treated with high doses of E, perfusions were conducted without the presence of inhibitors. Meanwhile, in other groups , the perfusions were conducted as follows: 1- perfusion without any inhibitor 2- perfusion with glibenclamide 3- perfusion with DIDS 4- perfusion with ACTZ 5- perfusion with EIPA. Six rats were used in each group.

*: Another group that was used in this study is 0.2µg estrogen+ peanut oil, each for 3 days, (this treatment was used to specifically evaluate the inhibitory effect of progesterone on the expression of CFTR and SLC26A6). We were only able to load 7 groups during western blotting, thus in chapter 4 this group replaced 2µg estrogen for qPCR, WB and IHC. No perfusion was conducted for this group.

### 3.5.2 Animal and surgical procedures

#### *3.5.2.1 Anaesthesia*

In this study animals were anaesthetized with ketamine (80 mg/kg) and xylazine (8mg/kg) injected intraperitoneally (IP). Ketamine is hydrochloric salt and is used for induction and maintenance of general anaesthesia, in combination with a sedative such as xylazine. Xylazine is a drug used for sedation, anaesthesia, muscle relaxation and analgesia in animals. The depth of anaesthesia was monitored by the loss of plantar reflexes while breathing movement was maintained.

#### *3.5.2.2 Ovariectomy*

Rats were anesthetized prior to ovariectomy which is used to eliminate endogenous steroid hormones. During surgery, a heat pad was used to maintain body temperature and vital signs were regularly monitored. 90% ethanol was used to sterilize all surgical instruments to reduce contamination.

Following anaesthesia rats were placed in left lateral position. Ovaries are located at caudal end of the ribs on lateral side of the animal. The area was shaved and swabbed with 90% ethanol to completely remove the fur. A small incision was made in the skin, after removing connective tissue where a second incision was made through the muscle; ovary can be seen between fats at the peritoneal cavity. The ovary was removed and remaining part of the oviduct and uterus was pushed back into peritoneal cavity. The incision in muscle and skin was sutured and same procedure was repeated for the left ovary (Parhizkar, *et al.*, 2008). After surgery, the animals were given intramuscular injection of 0.1 ml of Kombitrim antibiotic to prevent any post-surgical and wound infection.

### *3.5.2.3 Administration of sex steroid*

10 days after ovariectomy, rats were treated with 4 mg progesterone, (1mg progesterone dissolved in 0.1 ml peanut oil) or 0.2 µg 17-β estradiol, (dissolved in 0.1 ml peanut oil) which injected subcutaneously behind the neck. Control animals were treated with same volume of peanut oil (vehicle) (Salleh, *et al.*, 2005). Some experimental groups received higher dosage of E (2, 20 and 50µg) similar to that used in oral contraceptive pills (Petitti, 2003). One group was treated with 0.2µg oestradiol (3 days) +4mg progesterone (3 days), to roughly mimic steroid trend during oestrous cycle. Treatments were carried on for 3 days in all study groups. One day after treatment the rats subjected to perfusion.

### *3.5.2.4 Identification of stages of the oestrous cycle*

There is fluctuation in ovarian hormone levels during the female reproductive cycle (Figure 3.1). The oestrous cycle is 4-5 days in the rat and includes proestrus, oestrus, metestrus (early diestrus), and diestrus. Table 3.2 lists the characteristics of each stage (Islam, *et al.*, 2008).

The determination of the stages of the oestrous cycle was achieved by observation of vaginal smear under light microscope. Vaginal secretion was collected with a plastic pipette filled with 10 µl normal saline (NaCl 0.9%) and smoothly inserted into the rat vagina. The vaginal fluid was placed on the slide after collection, unstained material was observed under light microscope and proportion of different cell type was used to determine each stage. Three types of cells could be seen: round and nucleated cells are epithelial cells that is in proestrus stage; irregular cells without nucleus are the cornified cells can be seen in oestrus; the small round ones are leukocyte which is in high amount at diestrus and at metestrus the proportion of three different cells are similar (Islam, *et al.*, 2008; Marcondes, *et al.*, 2002). Following determination the stages of oestrous cycle, perfusion was carried out.

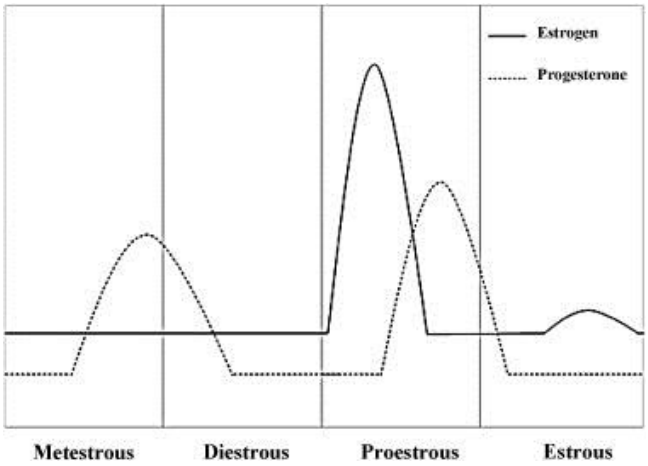



**Figure 3. 1** Hormonal profiles during the female reproductive cycle. Oestrogen level is high at early proestrus and estrus, while at diestrus progesterone increase.
**Source:** (Simpson & Kelly, 2012)

**Table 3. 2** Characteristic of different stages of the oestrous cycle, in rat this cycle is 4 to 5 days. Adapted from (Islam, *et al.*, 2008; Westwood, 2008)

| Stage of the oestrous cycle | Characteristics |
|---|---|
| proestrus<br>(12-13 hours in rat) | According the species one or several follicles start to grow<br>Last 1days-3 weeks<br>Endometrium develop<br>Female is not sexually receptive |
| estrus<br>(25-27 hours in rats) | Female is sexually receptive<br>Estrogen secretion exert the biggest influence<br>Ovulation |
| metestrus<br>(early diestrus)<br>(6-8 hours in rats) | Sign of estrogen reduce<br>Corpus luteum start to form<br>Progesterone secretion is very low<br>1-5 days |
| Diestrus<br>(55-57 hours) | Activity of corpus luteum ,secrete progesterone<br>If pregnancy does not occur, uterus reorganised for next cycle |

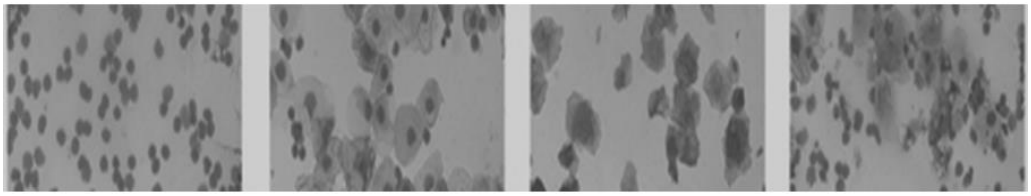



**Figure 3. 2** Identification ofthe oestrous cycle under light microscope.

## 3.6 Perfusion

Uterine perfusion is used to collect uterine fluid in order to measure its pH and ionic content. Uterine flushing can be used to collect the fluid, but it results in tissue damage and contamination of uterine fluid with interstitial fluid (Milligan & Martin, 1984), while the fluid volume is low (Armstrong, 1968; Salleh, *et al.*, 2005).

To perform uterine perfusion, the rats were brief anesthetized with IP injection of ketamine and xylazine HCl and maintained under anesthesia during the experiment. In this study only one horn was perfused in all groups and the other horn was collected for western blotting, IHC and qPCR. After anaesthesia, a small incision was made on a flank to expose the distal end of the uterine horn. An inflow prefilled fine polythene tubing of 15cm (ID 0.38mm, OD 1.09mm) was inserted into the uterine lumen at the uterotubal junction and tied there. The outflow tubing, 10 cm, with same diameter was inserted into the uterocervical junction and tied there after the ventral incision was made.

The cannula had minimal insertion into the lumen but had a slightly heat-flared ending to prevent them being readily pulled out by any ongoing uterine contractions. The Standard Infusion Harvard Pump 11 Plus Syringe (Hill Road Holliston, Massachusetts, USA), was used to deliver perfusion medium at constant rate of 1.3µl/min. A single sample of uterine fluid was collected into small, pre- weighed polythene tubes for a period of 3 hours as (figure 3.3).

### 3.6.1 Preparation of perfusion buffer

The composition of uterine perfusion fluid is based on published data showing that uterine luminal fluid has low $Na^+$ and high $K^+$ and contains NaCl, $Na_2HCO_3$, $Na_2HPO_4$, KCl, $MgSO_4$, HEPES, $CaCl_2$, glucose (Aguilar & Reyley, 2005; Salleh, *et al.*, 2005).

The concentration of $Na^+$ in the uterine fluid is approximately 124mEq/l in intact rat (Nordenvall, *et al.*, 1989) and human during the follicular phase of the menstrual cycle (Clemeston, *et al.*, 1970). $Na^+$ content of uterine are determined to be higher under the influence of E (128mEq/l) compared with P (119mEq/l) and control group (100mEq/l) (Tantayaporn, *et al.*, 1974). Nordenvall (1989) reported P administration increased $Na^+$ concentration (from 124 to 134 mEq/l).

The $K^+$ concentration in uterine luminal fluid is higher than plasma and it is affected by the hormonal status of animal. For example, its value within the oestrous cycle is 35.3mmol/l, in pseudopregnancy 46.2 and during implantation 46.8 (Clemeston, *et al.*, 1970). However Nordenvall (1989) reported that $K^+$ concentration was 22.3$\pm$2.4mEq/l during rat oestrus cycle which is less than earlier report. These differences could be due to different fluid collection methods whereby Clemeston (1970) used either uterine flushing or ligated uterine horns, and Nordenvall used microdialysis.

Based on these observations the following composition of perfusate was selected: 110.0 NaCl mmol/L, 14.3 $Na_2HCO3$, 1.0 $Na_2HPO_4$ (total $Na^+$ concentration= 125.3), 15 KCl, 0.8 $MgSO_4$, 10.0 HEPES, 1,8 $CaCl_2$ and 5.5 glucose with pH=7.34 (Salleh, *et al.*, 2005).

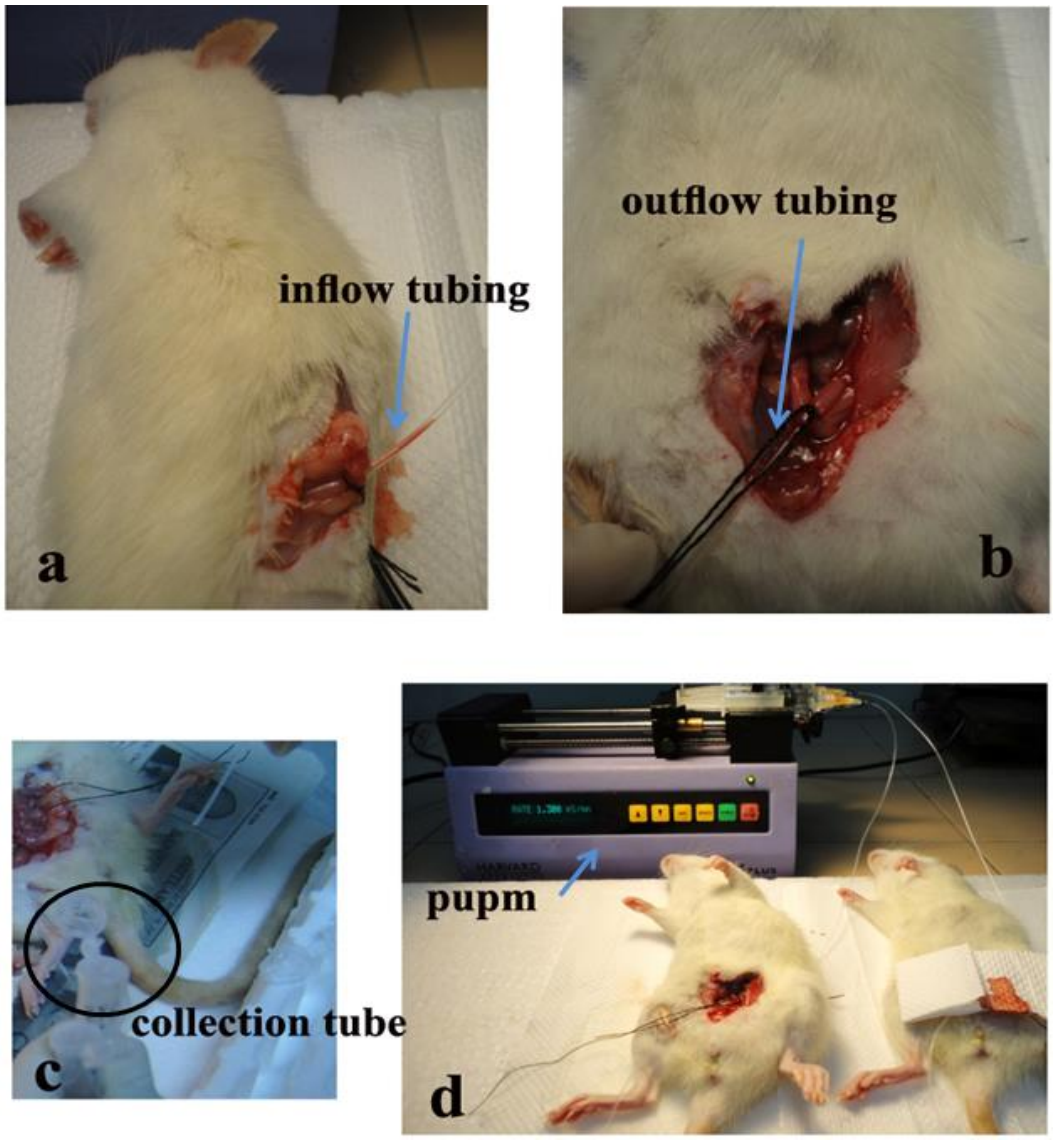



**Figure 3. 3** Procedure of uterine perfusion. Inflow tubing (a) was inserted into the uterine lumen at uterotubal junction, and outflow tubing (b) was put into the uterocervical junction. The uterine fluid was collected into the eppendorf tube (c). Infusion pump (d) delivered the buffer with constant rate during perfusion.

### 3. 6.2 Inhibitors

To investigate the role of carbonic anhydrase (CA), CFTR, $HCO_3^-$ transporter and NHE on the pH, volume and ionic content of the uterine luminal fluid, a related inhibitor was added to perfusate solution in different experimental group in table 3.1. After 3 hours collection of the uterine fluid, pH, volume and ionic content ($Na^+$, $Cl^-$, $HCO_3^-$) were monitored and contribution of ionic channel and enzymes on the composition of the uterine fluid composition was determined.

#### *3.6.2.1 Glibenclamide*

5-Chloro-N-[4-(cyclohexylureidosulfonyl) phenethyl]-2- methoxybenzamide,glyburide, N-p-[2-(5-Chloro-2- methoxybenzamido) ethyl] benzenesulfonyl-N′-cyclohexylurea (Table 3.3), is CFTR inhibitor. Glibenclamide, also known as glyburide, is an anti-diabetic drug, and is used in the treatment of type II diabetes. Glibenclamide is a sulfonylureas, and is able to inhibit CFTR, $Cl^-$ channels in epithelial cell (Hwang & Sheppard, 1999; Yamazaki & Hume, 1997). In this study, glibenclamide with concentration of 200 μM (Wang, *et al.*, 2003a) was used to inhibit CFTR activity.

#### *3.6.2.2 DIDS*

4′-Diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate (DIDS), (Table 3.3), is used as an anion inhibitor, and is able to inhibit $Cl^-/HCO_3^-$ exchanger and NBC. DIDS is one of the most effective members of stilbene disulfonates, which are potent inhibitors of inorganic anion exchanger (Poole & Halestarp, 1991). DIDS is also able to inhibit the voltage dependent CFTR $Cl^-$ channel (Hwang & Sheppard, 1999) through the intracellular pathway not extracellular (Zhang, *et al.*, 2000). DIDS concentration in this study was 500 μM.

### *3.6.2.3 EIPA*

5-(N-Ethyl-N-isopropyl) amiloride (EIPA), (Table 3.3), is selective blocker of $Na^+/H^+$ antiporter. EIPA is amiloride derivative. EIPA binding affinity for different NHE isoform is as follows : NHE1>NHE2>NHE5>NHE3 (Masereel, *et al.*, 2003). EIPA concentration was 100μM in this study (Brown, *et al.*, 2003).

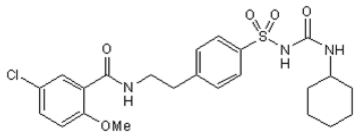
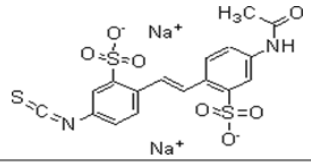
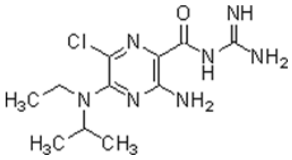
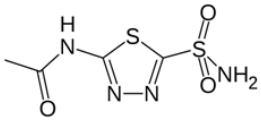
### *3.6.2.4 Acetazolamide*

C4H6N4O3S25-Acetamido-1,3,4-thiadiazole-2-sulfonamide,N-(5-Sulfamoyl-1,3,4-thiadiazol-2-yl) acetamide, N-(5-[Aminosulfonyl]-1,3,4-thiadiazol-2-yl) acetamide (ACTZ), (Table 3.3), a membrane permeable CA inhibitor (Berg, *et al.*, 2004). It is able to inhibit cytosolic CA in the uterus. CA catalyzes the reversible reaction:

$$CO_2 + H_2O \xleftrightarrow{CA} H_2CO_3 \xleftrightarrow{CA} H^+ + HCO_3^-$$

In above reaction, $CO_2$ and $H_2O$ produce $H_2CO_3$ and finally $H^+/HCO_3^-$ which is reversible. All the above reactions are mediated by CA and ACTZ are able to inhibit this reaction. The ACTZ is known under the trade name Diamox, used for the treatment of different diseases such as glaucoma, epileptic seizures, benign intracranial hypertension (pseudotumor cerebri), altitude sickness, and it is known as a diuretic. In glaucoma, for example ACTZ application decreases fluid formation in the eye and thereby reduces intraocular pressure. In this study ACTZ was used to inhibit CA activity in uterus with concentration of 100 μM (Breton, *et al.*, 1998).

**Table 3. 3** Formula, molecular weight (MW) and structure of different inhibitors

| inhibitor | structure |
|---|---|
| Glibenclamide<br>C23H28ClN3O5S<br>MW: 494 KD |  |
| DIDS<br>C16H8N2Na2O6S4 · xH2O<br>MW: 498.48 KD |  |
| EIPA<br>C11H18ClN7O<br>MW: 299.76 KD |  |
| Acetazolamide<br>C4H6N4O3S25<br>MW: 222.25 KD |  |

### 3.6.4 Sample analysis

#### *3.6.4.1 pH measurement*

The pH of collected uterine fluid was measured by HI 8424 NEW pH meter from Hanna instrument (Woonsocket, RI, USA). It is micro pH meter and is suitable for small sample size. For measuring the pH, first electrode was calibrated at pH=4 and pH=7, then the sample was introduced to electrode.

#### *3.6.4.2 Fluid secretion rate*

In order to measure the fluid secretion rate, the net weight of the collected fluid was divided by the total perfusion time (180 minutes).

### *3.6.4.3 Measurement of $HCO_3^-$*

All ionic measurement was performed by used of ADVIA Chemistry (Siemens, USA). $HCO_3^-$ was quantified by enzymatic assay with the ADVIA Chemistry Carbon Dioxide Liquid ($CO_2$-L) method based on the phosphoenolpyruvate carboxylase (PEPC) catalyzed reaction of $HCO_3^-$ with phosphoenol pyruvate to produce oxaloacetate. Malate dehydrogenase (MDH) is used to catalyze the indicator reaction in which the amount of reduced nicotinamide adenine dinucleotide (NADH) analog, was oxidized with the decreased absorbance at 410/478 nm, while is proportional to the amount of $CO_2$ in the sample.

### *3.6.4.4 Measurement of $Na^+$*

The ADVIA $Na^+$ method is based on an indirect potentiometric procedure using an ion selective electrode (ISE). The $Na^+$ ISE responds selectively to $Na^+$ ions according to the Nernst equation. The sample is mixed with ISE buffer, thereby providing a constant pH and a constant ionic strength solution. As the buffered sample moves through the ion selective electrode, changes in the electrical potential take place. These electrical potential changes are measured against the potential of a reference electrode in order to derive the correct analog value for that sample.

### *3.6.4.5 Measurement of $Cl^-$*

The ADVIA $Cl^-$ method is based on an indirect potentiometric procedure using an ion selective electrode (ISE). The $Cl^-$ ion selective electrode responds selectively to $Cl^-$ ions according to the Nernst equation.

For each ionic measurement 100µl of sample has been used.

## 3.7 Protein expression

### 3.7.1 Western blotting

#### *3.7.1.1 Principle of the study*

Western blots which are known as a protein immunoblot, are performed to determine the molecular weight of a targeted protein and to estimate relative amounts of the protein present in different samples.

The first step is to separate the proteins by gel electrophoresis (SDS-page), then antigen is transferred to the blotting membrane electrophoretically. Following antigen binding to the membrane, the remaining protein binding site on the membrane is blocked to reduce nonspecific protein binding. Next primary antibody is added to react with the protein of interest. After reaction of target by primary antibody, the membrane is incubated with secondary antibody linked with horse radish peroxidase, which allows interaction between primary and secondary antibody. Finally, the target protein is visualized with incubation of membrane with substrate detection reagent. The substrate cleaved the horse radish peroxidase linked to the secondary antibody and produce colorful band (Walker, 1996).

#### *3.7.1.2 Sample collection*

In each study group, (described in table 3.1) after uterine perfusion, the intact horn of uterus was removed. The organs were immediately frozen in liquid nitrogen (-196ºC), samples was transferred to -80°C for long term use.

#### *3.7.1.3 Protein extraction*

Protein extraction was achieved with the usage of PRO-PREP protein extraction solution, which is able to extract protein from whole cell, nucleus, membrane and cytoplasm. The buffer contain highly denaturing ionic detergent such as sodium

dodecyl sulphate (SDS), lithium dodecyl sulphate and sodium deoxycholate that isolate protein as a monomeric form for western blotting and molecular weight analaysis; and non-ionic detergent, such as Triton X-100 and applied in protein – protein interaction. It also include zwitterioninc detergent CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1- propanesulfonate ) that is more effective in disrupting protein-protein interaction.

The work flow was as follow:

1- 30mg of uterus was cut and soaked in 600µl of extraction solution.

2- The tissue was homogenized with ultrasonic cell disruptor (Branson) to disrupt the tissue; it was placed on ice.

3- The sample was then kept at -20°C for 30 minute to induce cell lysis, after that second round of homogenization was performed and sample transferred to -20°C for 30 more minutes.

4- The sample was centrifuged at 13000rpm, 4°C, 10minutes, and the supernatant was transferred to new 1.5ml tube and placed in -20°C.

#### *3.7.1.4 Determination of the protein concentration*

Protein concentration was determined by Micro BCA Protein Assay Kit. The kit includes micro BCA reagents and albumin standard ampule which contains Bovine Serum Albumin (BSA) at 2mg/ml in 0.9% saline and 0.05% sodium azide. The protein assay kit is a detergent-compatible bicinchoninic acid formulation for the colorimetric detection and quantitation of total protein. Bicinchoninic acid (BCA) is the detection reagent for $Cu^{+1}$, which is formed when $Cu^{+2}$ is reduced by protein in an alkaline environment. A purple-colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion $Cu^{+1}$. This water-soluble complex exhibits a strong absorbance at 570 nm that is linear with increasing protein concentrations.

The protein concentration was calculated from a linear plot of BSA standard concentration (on the x axis) of the intensity of color changes absorbance obtained from the reaction between BSA and BCA reagent. BSA standard was prepared at concentrations of 200, 40, 20, 10, 5, 2.5, 1, 0.5 and 0 µg/ml. Tissue samples were diluted 1 in 5 with PBS. 50 µl of sample or standard was added to 50 µl of BCA reagent in a 96 well plate in triplicate. The plate was incubated at 37ºC for 2 hours, after while the intensity of color changes in each well was qualified by a plate reader at 570nm. Protein standard curve was generated and the concentration (µg/ µl) of samples calculated from the standard curve.

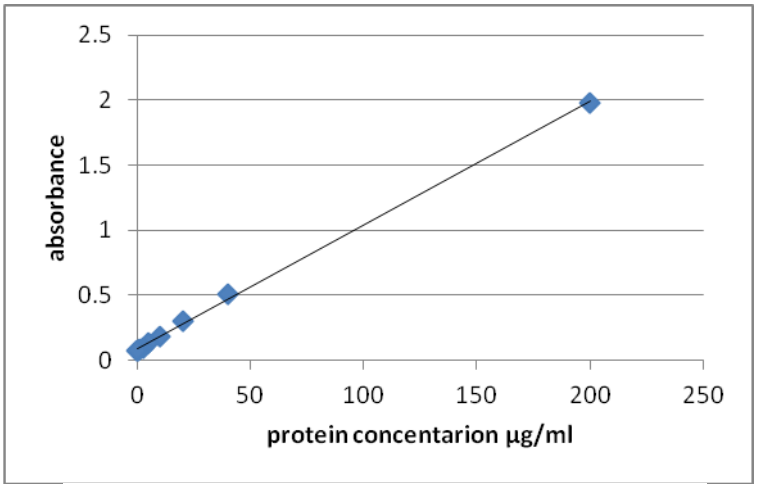



**Figure 3. 4** Standard curve for protein determination.

### *3.7.1.5 Principle of SDS-PAGE*

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) is a useful technique for detection, quantification, and characterization of a specific protein (Pettegrew, *et al.*, 2009). Migration of protein in electrophoresis is based on its charges, mass, size and strength of electrical fields.

The matrix is polyacrylamide gel which is polymer of acrylamide and bis-acrylamide (both from Sigma, USA). The presence of N,N,N',N'-tetramethylethylenediamine (TEMED), and ammonium persulfate (APS), catalyzes the gel polymerization (Walker,

2007). The separation of molecule within the gel is determined by size of the pores that formed within the gel. Low percentage of polyacrylamide gel is suitable for high molecular weight protein while increase in polyacrylamide percentage is appropriate matrix for low molecular weight protein.

SDS is an ionic detergent which is used in polyacrylamide gel. SDS denatures the protein and makes them negatively charged. The charge is equally distributed to the protein molecule based on the protein length that leads to migration of protein according their size.

### *3.7.1.6 Running Western blotting*

#### *3.7.1.6.a Running Polyacrylamide gel*

Polyacrylamide consist of two different gel formulation, first is resolving gel which used at the bottom of gel caster with a pH at 8.8. In this study resolving gel with 12% (for CAXII, NHE 1, NHE2, NHE4, CFTR, SLC26A6 and NBC) and 15% (CAII) was used. The next gel is placed above the resolving gel is stacking gel 4% at pH 6.8 and is used to pack protein together after loading. Table 3.4 shows the composition of each gel. The gel in the gel cast was kept overnight at 4°C.

The following day, the gel was placed inside the electrophoresis tank and bathed in migration buffer. 10µg protein with an equal volume of loading buffer was mixed and boiled for 5minutes, then centrifuged for 10 second. Samples were loaded to each well. Prestained protein marker was loaded into one of the empty wells to provide the protein size marker. Rabbit polyclonal β actin (Abcam, UK) was used as a loading control, to check whether all samples have been loaded in lanes carefully. In addition it was used to compare the expression between different samples.

Electrophoresis carried out at constant voltage, 100 volt and the power supply was switched off when the front dye reached bottom of the gel.

**Table 3. 4** Composition of 15% , 12% resolving gel and 4% stacking gel.

| Stock solution | Resolving gel 15% | Resolving gel 12% | Stacking gel 4% |
|---|---|---|---|
| Acrylamide | 8.3ml | 10ml | 0.665ml |
| 4X resolving gel | 4ml | 4ml | - |
| 4X stacking gel | - | - | 1.25ml |
| 10% SDS | 0.2ml | 0.2ml | 50µl |
| 10% APS | 100µl | 100µl | 25µl |
| TEMED | 6.75µl | 6.75µl | 2.5µl |
| Double distilled H2O | 6.4ml | 4.7ml | 3ml |

*3.7.1.6.b Transfer of protein to membrane for western blotting*

After protein separation by electrophoresis, the protein must be transferred to PVDF membrane for immunoblotting. PVDF membranes, are hydrophobic, hence it was necessary to prewet the membrane in 100% methanol (2minutes) so it can be used with aqueous buffer. After a while, the membrane was soaked in towbin buffer (transfer buffer) which contain 20% methanol. The presence of methanol in the transfer buffer is to promote dissociation of SDS from the protein and improves adsorption of proteins onto membranes in the presence of SDS (Pettegrew, *et al.*, 2009). The membrane was placed in contact with gel and air bobbles were removed carefully.

The gel-membrane sandwich was placed in transfer tank for electro blotting. Blotting was carried out at constant voltage of 100 volts for 90minutes. Following the transfer of proteins to membrane, the membrane was washed for 5 minutes.

*3.7.1.6.c Blocking*

The next step is to block the membrane which allows maximize signal noise ratio. 5% of BSA of 60 minute (for CAII) and 90 minutes for other target were used in this step. Washing with PBST (phosphate buffer saline- tween 20), was done 3 times each for 5 minutes; it is necessary before incubation with primary antibody.

*3.7.1.6.d Incubation with primary antibody*

The primary antibody was diluted 1:1000 in PBST buffer. Incubation with primary antibody was carried out for 90minutes at room temperature (Table 3.5).

*3.7.1.6.e Incubation with secondary antibody*

After incubation with primary antibody, the membrane was washed 3 times. The secondary antibody is attached to horseradish peroxidase (HRP), was diluted 1:2000. Incubation with secondary antibody was at room temperature for 60minutes (Table 3.5).

*3.7.1.6.f Visualizing*

The final step in the western blotting is to visualize the proteins, which achieved by Opti-4CN™ Substrate Kit. This is colorimetric (HRP) substrate; detected band can be seen by necked eyes.

**Table 3. 5** Primary and secondary antibodies which has been used in this part.

| Target protein | Primary antibody | Secondary antibody |
|---|---|---|
| CAII | Mouse monoclonal | Anti mouse, hrp |
| CAXII | Rabbit polyclonal | Anti rabbit hrp |
| NHE1 | Goat polyclonal | Anti goat hrp |
| NHE2 | Goat polyclonal | Anti goat hrp |
| NHE4 | Goat polyclonal | Anti goat hrp |
| CFTR | Goat polyclonal | Anti goat hrp |
| NBC | Rabbit polyclonal | Anti rabbit hrp |
| SLC26A6 | Goat polyclonal | Anti goat hrp |

*3.7.1.6.g Analysis of western blot result*

After the visualization of target protein, the membrane was scanned by a gel documentation system (Vilber Lourmat, from Fisher Scientific, USA). The density of bands was determined by ImageJ (National Institutes of Health, USA). The ratio of target bands over β actin of each sample represents the relative expression of target proteins.

### 3.7.2 Immunohistochemistry (IHC)

#### *3.7.2.1 Principle*

IHC is used to identify the specific location of antigen (target protein) in the tissue, by application of primary antibody and biotinylated secondary antibody, this complex visualized with DAB (diaminobenzidine tetrahydrochloride) staining. In this study adult female rats were killed by cerebral dislocation after perfusion, and intact uterine horn was removed and immediately immersed in 4% paraformaldehyde before tissue processing.

#### *3.7.2.2 Method*

*3.7.2.2.a Principle of tissue processing*

Tissue processing is necessary to eliminate water from the tissue and replace it with a medium that allow cutting of thin sections. Fixation was carried out for 4-5 hours, as longer fixation time might destroy antigens in the tissue. Fixation preserves cell and tissue structure close to a real situation, with inhibition of autolysis and bacterial activity. Thus fixation that maintain tissue structure and proteins (Meyer & Hornickel, 2010), is the first and important step in IHC.

In this protocol, paraformaldehyde was used to fix the tissue structure. Use of paraformaldehyde by cross linking protein, does not destroy the protein structure and preserve the protein antigenicity (Meyer & Hornickel, 2010).

After tissue fixation, post fixation and preparatory process were performed, and include dehydration, clearing, infiltration and embedding.

Dehydration, dissolves various cellular component, for example water soluble protein dissolved in lower aqueous alcohols. To achieve dehydration, specimen goes through increasing alcohol concentration.

Next step was clearing, which is an intermediate stage from dehydration to infiltration and embedding. Dehydrants are usually insoluble in paraffin wax, hence it is essential to apply a solvent that is compatible with both dehydrant and embedding medium. Chloroform, is an example of a clearing agent.

Subsequently, infiltration with wax is performed to saturate the tissue cavities and cells. The last step is embedding with paraffin, a medium that provides an essential supporting medium for microtome sectioning.

Protocol for tissue processing

1- Fixation in 4% paraformaldehyde for 4-5 hours.
2- Incubation in 70%ethanol for more than 2 hours or overnight.
3- Incubation in 90% ethanol for 2 hours.
4- Incubation in absolute ethanol twice for 2 hours.
5- Incubation in chloroform overnight to clear any remaining ethanol.
6- Incubation in 4 changes of paraffin wax each 45minutes.
7- Final step was embedding of tissue into fresh wax. The specimens can be maintained in 4°C until sectioning.

*3.7.2.2.b Sectioning*

After tissue processing, tissues were cut into 5 µm section by microtome. Then sections were floated in water and mounted on polysine slides, finally slides were dried at 37°C in an oven, overnight.

*3.7.2.2.c Deparaffinization and tissue hydration*

Before slides were stained, hydration (with decreasing ethanol grade) was carried out by the following steps:

(i) Deparaffinization with 3 changes of xylene each one 5 minutes.

(ii) Washing in 100% ethanol 2 times each 10 minutes.

(iii) Washing in 95% ethanol twice each 10 minutes.

(iv) Washing in PBS twice each 5 minutes.

(v) Washing in deionized water for 1 minute.

*3.7.2.2.d Antigen retrieval*

Antigen retrieval or antigen recovery is used to unmask antigen which masked during fixation. Antigen recovery increases reactivity of antigen within specimens. It was performed by these steps:

(i) Preparing 10mM sodium citrate buffer, pH 6.

(ii) Immersing slides in a container with sodium citrate buffer.

(iii) Heating at 95°C for 5 minutes.

(iv) Slides allowed cooling in buffer for 20 minutes.

(v) Slides washed in deionized water, 3 times each 2 minutes.

Prior to immunostaining, it is necessary to block the endogenous peroxidase activity with incubation in 0.1-1% hydrogen peroxidase in deionized water for 5-10 minutes.

*3.7.2.2.e Immunostaining*

In this step, rat ABC staining system was used. The kit contained: 1ml normal blocking serum, 250µg biotinylated secondary antibody, 0.5ml avidin and 0.5ml biotinylated horseradish peroxidase (AB reagent), 1 ml 50x peroxidase substrate, 1 ml 50x 3,3' DAB chromogen, and 3 ml 10x substrate buffer.

Preparation of working solutions:

(i) Blocking serum: combine 75µl normal blocking serum diluted with 5 ml PBS (phosphate buffer saline).

(ii) Biotinylated secondary antibody: 75µl normal blocking serum mixed with 5 ml PBS and 25 µl biotinylated secondary antibody.

(iii) AB enzyme reagent: 50 µl reagent A (avidin), plus 50 µl reagent B (biotinylated HRP) and 2.5 ml PBS, prepared 30 minutes before using the mixture

(iv) Peroxidase substrate: 1.5 ml distilled H2O, and 5 drop 10x substrate buffer plus1 drop DAB chromogen and 1 drop 50x peroxidase substrate

The sections went through the following steps:

(1) Incubation in 1.5% blocking serum in PBS for one hour.

(2) Incubation with primary antibody (Table 3.5) overnight at 4°C. Optimal concentration of antibody was 1:100 diluted in 1.5% blocking serum in PBS.

(3) Washing with PBS 3 times each 5 minutes.

(4) Incubation with biotinylated secondary antibody.

(5) Washing with PBS 3 times each 5 minutes.

(6) Incubation with AB reagent for 30 minutes.

(7) Washing with PBS 3 times each 5 minutes.

(8) Incubation with 1-3 drops of peroxidase substrate for 10 minutes.

(9) Washing with deionized H2O, 5 minutes.

(10) Counterstaining in hematoxilyn for 5-10 seconds, then immediately washing the section with several changes of deionized water.

Throughout the study, nonspecific immunoglobulin (same species with related primary antibody) was used as a negative control to determine the specificity of primary antibody.

(11) Dehydration the specimen with 2 times 95% ethanol for 10 seconds each; 2 times with 100% ethanol for 10 seconds each; 3 times xylenes for 10 seconds each and wipe off excess xylenes.

One to two drops of mounting medium was added and the section covered with a glass coverslip.

The slides were ready to observe by light microscope. The secondary antibody was according Table 3.6.

**Table 3. 6** The secondary antibodies that has been used for IHC.

| Target protein | Secondary antibody |
|---|---|
| CAII | goat anti mouse |
| NHEs, CFTR, SLC26A6 | bovine anti goat |
| CAXII, NBC | goat anti rabbit |

### *3.7.2.3 Evaluation of immunostaining*

The relative intensity of the products of immunoreaction at the luminal and glandular epithelia of the uterine sections were evaluated and graded blindly by three independent observers by using a light microscope (Olympus, Japan) at 10X and 100X magnifications. The staining intensity was estimated semi-quantitatively on a scale of 0–3+ (+++) as: −, no detectable stain; −/+, faint; +, moderate; ++, strong; and +++, very intense staining, as were previously described (Perrot-Applanat, *et al.*, 1994)

### 3.7.3 Real time PCR

#### *3.7.3.1 Principle*

Quantitative real time PCR (qPCR) is a sensitive technique to evaluate gene expression in which data is collected throughout the PCR amplification process as it occurs. It combines amplification and detection of the PCR product into a single step. Real time PCR can produce accurate data, does not necessitate post-amplification manipulation such as gel agarose by use of development of fluorogenic labelled probes, and can even detect a single copy of a specific transcript (Palmer, *et al.*, 2003). It is able to detect small differences in gene expression between samples, and it needs less mRNA template in comparison with other methods. However it requires expensive equipment and reagents (Wong & Medrano, 2005).

The mRNA quantification by real time PCR can be achieved in a one-step or two step reaction. In one step method, the whole reaction from cDNA synthesis to PCR amplification is performed in a single tube. This method may produce better results for detection of rare transcripts. In two-step reaction reverse transcription and PCR amplification occur in separate tubes which allow storage of cDNA for longer time (Wong & Medrano, 2005). In this study one step PCR was performed.

In any of these methods, quantitation is either absolute quantitation in which serially diluted standards of known concentrations is used to generate a standard curve and determine the concentration of unknown sample; or relative quantitation, that measure alteration in gene expression based on external standards or reference samples (Livak & Schmittgen, 2001).

Real time PCR is based on detection of fluorescence while PCR occurs. Two types of chemistries are used to detect the PCR:

1- TaqMan chemistry (known as “fluorogenic 5′ nuclease chemistry”), which uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR cycles. TaqMan assay was used in this study.

2- SYBR Green I dye chemistry, is a highly specific, double-stranded DNA binding dye, to detect PCR product as it accumulates during PCR cycles.

Each PCR reaction is divided to 3 different phases, exponential, linear and plateau phase, (Figure 3.5-6) (Tichopad, *et al.*, 2003). During the first 10-15 cycles that PCR, fluorescence emission has not risen above background. In exponential phase product is accumulated and reaction is very specific and precise thus this phase is used for data collection and result analysis. At the linear phase the reaction become slower due to consumption of components; while at plateau phase the reaction stops and no more product has been produced (Bustin, 2000) .

### *3.7.3.2 Sample Collection*

For this study RNAlater® was used to preserve tissue’s RNA prior to extraction. RNAlater® is a RNA stabilization solution that rapidly permeates into the tissue and protects RNA from degradation. Tissue can be preserved indefinitely in RNAlater® solution at –20°C or below. To collect sample, the uterus was excised, and soaked in 5 volumes of RNAlater®.

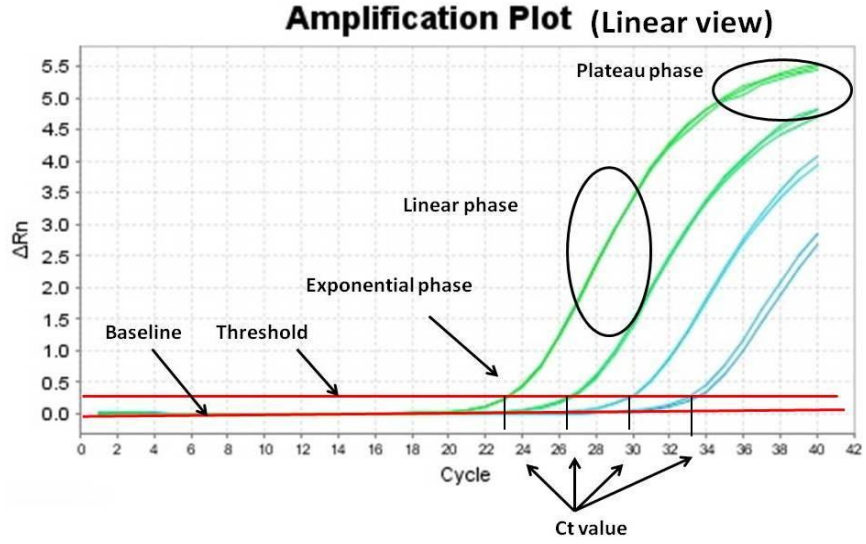



**Figure 3. 5** Baseline, threshold, Ct value in different view of amplification plot.

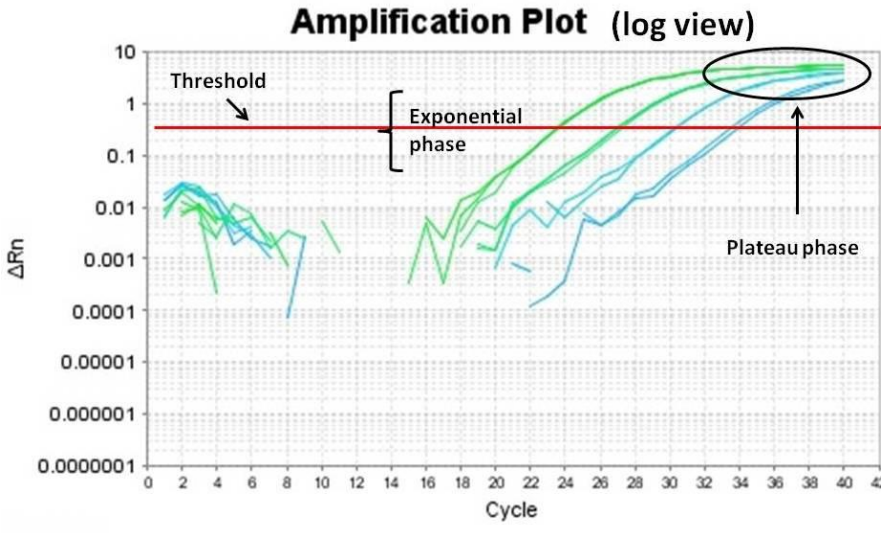



**Figure 3. 6** Baseline and Threshold. . Baseline, fluorescence cannot be detected by instrument and usually is between cycle 3-15. Threshold, is a level of normalized reporter signal that is used for $C_T$ determination in real-time assays. The level is set to be above the baseline but sufficiently low to be within the exponential growth region of an amplification curve. The cycle number at which the fluorescence signal associated with a particular amplicon accumulation crosses the threshold is referred to as the $C_T$.

### *3.7.3.3 Protocol for RNA Extraction*

For this study Ribo Pure kit, was used to extract RNA from the uterus. The kit contains: TRI Reagent, filter cartridges, collection tubes, wash solution, elution buffer. TRI Reagent is the improved version of the popular single-step method of total RNA isolation and combines phenol and guanidine thiocyanate in a monophasic solution to rapidly inhibit RNase activity. Before extraction, the tissue was retrieved from RNAlater® solution with sterile forceps and excess RNAlater® quickly was removed with an absorbent paper towel and then soaks the sample in RNA lysis buffer.

*3.7.3.3.a Homogenization*

100 mg of uterus was powdered in liquid nitrogen, followed by addition of 1 ml of TRI reagent added to the tissue powder. Homogenate then was incubated at room temperature for 5 minutes, and centrifuged at 12000g, 10 minutes at 4 ºC. The supernatant transferred to a new tube.

*3.7.3.3.b RNA extraction*

Two hundred µl chloroform was added to 1 ml of homogenate, mixed well and incubated for 5 minutes at room temperature (25 ºC). It was then centrifuged at 12000g, for 10 minutes at 4 ºC. The centrifuging at this step, led to separation of the phase of mixture. The lower phase was red phenol-chloroform phase, an interphase, and a colorless upper part in the aqueous phase. The first two phases contain DNA and protein, where upper phase contains RNA. At higher temperature than 4°C the DNA may sequester in the aqueous phase. 400 µl of aqueous phase was transferred to a new 1.5 ml micro centrifuge tube to proceed to RNA purification. The intermediate and lower phases can be used for DNA or protein extraction.

*3.7.3.3.c Final RNA purification*

RNA precipitation was achieved by adding of 0.5ml isopropanol per 1 ml of TRI reagent for initial homogenization, and mixed immediately. The mixture was incubated at room temperature for 10 minutes. The sample was passed through a filter cartridge and centrifuged at 12000g, 30 second at room temperature. The filter then was washed twice with 500 µl of wash buffer. Finally the cartridge was eluted with 100 µl of Elution buffer. Incubated at room temperature, 2 minutes and centrifuged for 30 seconds.

***3.7.3.4 RNA quality control***

*3.7.3.4.a Optical density*

The extracted RNA may be contaminated by protein, DNA, EDTA and phenol that can act as an inhibitor of RNA downstream applications. Thus, evaluating integrity and quality of extracted RNA is essential. RNA was quantified by Gene Quant 1300 spectrophotometer, from UK by diluting RNA in TE buffer. Then 2 µl of RNA was diluted with 78 µl of TE buffer while 80 µl of same buffer used as a blank. Reading was evaluated by A260/280, A260/230.

*3.7.3.4.b Detection of genomic DNA contamination*

Agarose gel is the most common way to evaluate RNA integrity. Gel electrophoresis is performed on 1% agarose in TBE buffer and 0.5µg/µl ethidium bromide for gel staining. After running the gel for 30 minutes in 110 V (20V/cm), gel was observed with a gel documentation system (Vilber Lourmat, from Fisher Scientific). Two sharp and clear bands at 2:1 ratio can be seen indicating 28 and 18S rRNA. Any smearing or shoulder of RNA indicates gross degradation. The ladder in RNA agarose gel electrophoresis was 1Kb DNA step ladder.

Presence of band above 28S can indicate RNA smearing or DNA contamination. To assess coextraction of DNA with RNA, PCR can be run without reverse transcriptase. In

this case PCR amplification without RT is due to presence of genomic DNA. However if the assay probe spans exon-exon junction of the associated genes, it will not detect genomic DNA. In applied biosystem this type of assay labeled with (**m**) as what used in this research.

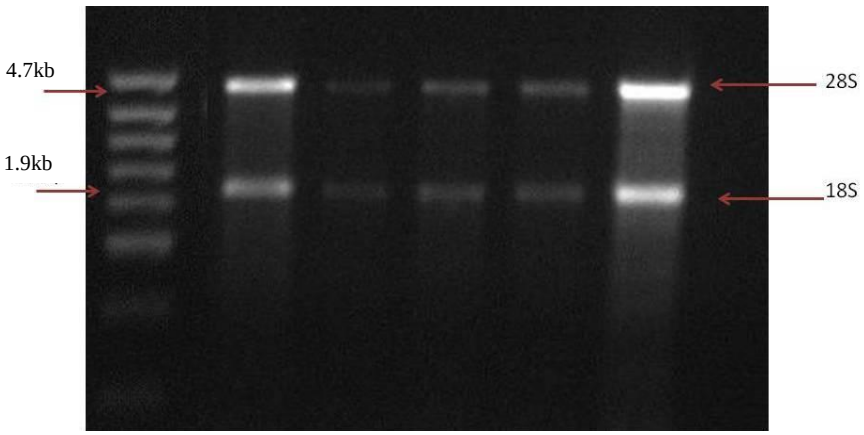



**Figure 3. 7** RNA gel electrophoresis.

*3.7.3.4.c Standard curve*

The real-time PCR data from standard curve plots can be used to identify if inhibition were presented in RNA. The standard curve also used to examine PCR efficiency. The slope of a standard curve is commonly used to estimate the PCR amplification efficiency of a real-time PCR reaction. A real-time PCR standard curve is graphically represented as a semi-log regression line plot of $C_t$ value vs log of input nucleic acid (The standard curve of different assays which has been used in this study is shown in appendixB).

Thus purified RNA that is qualifying for real time PCR must contain less than 0.005% of genomic DNA by weight, free of inhibitors of reverse transcription and PCR, dissolved in PCR-compatible buffer, free of RNase activity and in nondenatured state.

### *3.7.3.5 Justification for number and choice of reference genes*

Normalization of expression data is usually achieved by measuring the expression of several candidate reference genes (Table 3.7) in a number of representative samples, and selecting the gene(s) that show least variation as reference(s) for the particular study. To choose the best reference gene, 6 reference genes were screened for each group of 3 samples. GenEx, v5 (from Multid Analyses AB) used to evaluate the constancy or variability of reference gene expression.

The expression of reference gene was compared with the geNorm, and NormFinder application model of GenEx.

Both geNorm and NormFinder provide bar charts showing which gene are most stably expressed. geNorm select the pair of most stably expressed reference gene whereas NormFinder indicate that which reference gene might be optimal and in fact select the almost same order of stability as geNorm.

NormFinder is first described by Anderson (2004), is the optimal normalization gene among a set of reference gene. It ranks reference genes according to their expression stability in a given set of sample and given experimental design. It estimates overall expression variation of the candidate normalization gene and the variation between sample (In this study control group vs other groups).

geNorm uses the same data as NormFinder but all samples are treated as being from a single population, geNorm sequentially eliminate the genes that shows the highest variation relative to all of the gene based on pair expression value in all study sample, the variation called M-value.

**Table 3. 7** Reference genes which has been used in this study to find out the suitable reference gene

| Reference gene symbol | Gene name | Assay id |
|---|---|---|
| Hmbs | hydroxymethylbilane synthase | Rn00565886_m1 |
| Actb | actin, beta | Rn00667869_m1 |
| Gapd | glyceraldehyde-3-phosphate dehydrogenase | Rn99999916_s1 |
| Hprt1 | hypoxanthine phosphoribosyltransferase 1 | Rn01527840_m1 |
| B2m | beta-2 microglobulin | Rn00560865_m1 |
| Ppia | peptidylprolyl isomerase A (cyclophilin A) | Rn00690933_m1 |

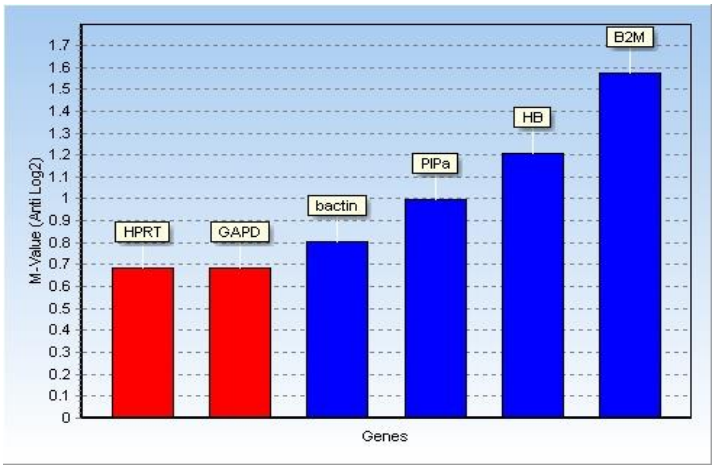



**Figure 3. 8** The lowest M-value belong to HPRT and GAPD

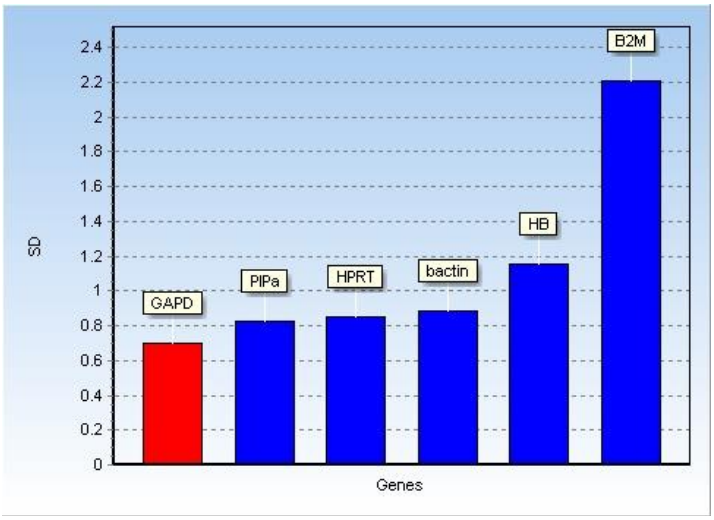



**Figure 3. 9** According Normfinder, GAPD is the best reference

### *3.7.3.6 Running real time PCR*

After RNA extraction and quantification, RNA was converted to cDNA for 2step real time PCR, or stored at -80ºC for 1step qPCR. In this study 1 step real time PCR was used to investigate the gene expression in different treatment groups by using of TaqMan® RNA-to-CT 1-Step Kit.

This kit contains two tubes:

1- TaqMan® RT-PCR Mix (2✕) contains:

– AmpliTaq Gold® DNA Polymerase, UP (Ultra Pure)

– dNTPs (dATP, dCTP, dGTP, dTTP, and dUTP)

– ROX™ passive reference

– Optimized buffer components

2- TaqMan® RT Enzyme Mix (40✕) contains:

– ArrayScript™ UP Reverse Transcriptase

– RNase inhibitor

To prepare the reaction cocktail, 50ng of total RNA in 1µl used as template. Each reaction tube contain 5 µl master mix, 0.25 µl reverse transcriptase, 3.25 µl RNase free water and 1 µl sample. Thus total volume was 10 µl per reaction. In the negative control well 1 µl RNase free water was used in place of sample.

In the reaction plate, 3 replicates of each sample along with 3 replicates of endogenous control were used. Expression of endogenous control is not affected by different experimental condition thus can be used to normalize fluorescence signals for target assay. In this study GAPD and Hprt1 were chosen after screening different reference genes.

After preparing the reaction plate, plate was loaded into Applied Biosystem Step One Plus thermal cycler.

Reverse transcription and PCR condition are shown below according TaqMan® RNA-to-CT 1-Step Kit was:

**Table 3. 8** Real time PCR running program

| Stage | Step | Temp | Time |
|---|---|---|---|
| Holding | Reverse transcription | 48°C | 15min |
| Holding | Activation of AmpliTaq Gold DNA polymerase, (Ultra Pure) | 95°C | 10min |
| Cycling (40 cycle) | Denature | 95°C | 15sec |
| | Anneal/Extend | 60°C | 1 min |

**Table 3. 9** TaqMan assays that were used

| Assay ID | Gene Symbol | Gene Name |
|---|---|---|
| Rn00570700_m1 | Car 2 | carbonic anhydrase 2 |
| Rn01418250_m1 | Car12 | carbonic anhydrase 12 |
| Rn00670440_m1 | Slc4a4 | solute carrier family 4 (anion exchanger), member 4 |
| Rn01455968_m1 | Cftr | cystic fibrosis transmembrane conductance regulator homolog |
| Rn00561924_m1 | Slc9a1 | solute carrier family 9 (sodium/hydrogen exchanger), member 1 |
| Rn00688610_m1 | Slc9a2 | solute carrier family 9 (sodium/hydrogen exchanger), member 2 |
| Rn01437220_m1 | Slc9a4 | solute carrier family 9 (sodium/hydrogen exchanger), member 4 |
| Rn01445892_m1 | Slc26a6 | solute carrier family 26, member 6 |

### *3.7.3.7 Analysis of result*

Data was analyzed according to comparative fold changes in expression (Wong & Medrano, 2005) where:

$\Delta Ct$= Ct (target gene)- Ct (reference gene)

$\Delta\Delta Ct$= $\Delta Ct$ (treated sample)- $\Delta Ct$ (untreated sample)

And Fold changes= $2^{-\Delta\Delta Ct}$

Measurements were normalized using the GenEx software. The relative quantity of target in each sample was determined by comparing normalized target quantity in each

sample to normalized target quantity in the reference. Data Assist v3 software from Applied Biosystems was used to calculate RNA folds changes.

### *3.8 Statistical analysis*

Results are expressed as mean±SEM. For the functional studies n=6 per group. For real time PCR, western blotting n=4 per group, and each experiment was performed in triplicate. Statistical analysis was carried out using SPSS 19. Comparison among groups was by one-way ANOVA, followed by Tukey's test. A p value $<0.05$ was considered statistically significant.

## Chapter 4

## Result & Discussion

# BICARBONATE TRANSPORTER

## 4.1 mRNA level, protein expression and localization of CFTR

### 4.1.1 CFTR expression in ovariectomized rats

In figure 4.1.A, the level of CFTR mRNA was significantly reduced following P treatment as compared to 0.2E and control. The inhibitory effect of P on CFTR mRNA expression could be seen in the group treated with 0.2E followed by P, in which there was a 13-fold reduction in the mRNA level as compared to 0.2E or E plus vehicle (0.2E + vehicle). As doses increase resulted in increase in CFTR mRNA following E treatment. The highest amount of CFTR mRNA was observed following treatment with 50E, which was 16-fold higher as compared to the control. Treatment with 0.2E resulted in a 12-fold increase in the CFTR mRNA level, (Figure 4.1.A).

Protein expression followed an almost similar pattern to the changes in mRNA (Figure 4.1.B). P treatment resulted in decreased CFTR protein expression as compared to the control and 0.2E. There was a dose-dependent increase in the protein expression under E, in which treatment with 50E resulted in approximately two-times increase in protein expression as compared to 0.2E. As expected, E treatment followed by three days of P treatment (E + P) resulted in a reduction in the protein expression by about 1.5 times as compared to 0.2E or E plus vehicle (0.2E + vehicle).

### 4.1.2 CFTR throughout the oestrous cycle

Similar patterns of CFTR mRNA changes could be seen throughout the oestrous cycle. Our finding, however, indicates that CFTR mRNA was the highest at proestrus, with a 10-fold increase, while at estrus, there was only 3-fold increase in its expression. In this study CFTR mRNA expression was detected even at an earlier stage of the oestrous cycle than the previous report (Chan, *et al.*, 2002). During diestrus stage, however, the mRNA level was noted to be the lowest with only a 1.5-fold increase as compared to the

control (Figure 4.1.A) .The highest expression of CFTR protein was noted at proestrus and estrus which correlates with a high plasma E level. The amount expressed declined at metestrus, reaching the lowest at diestrus. This is consistent with increasing level of P in the circulation (Figure 4.1.B). CFTR was observed as 90kDa protein on the blot (Figure 1.C).

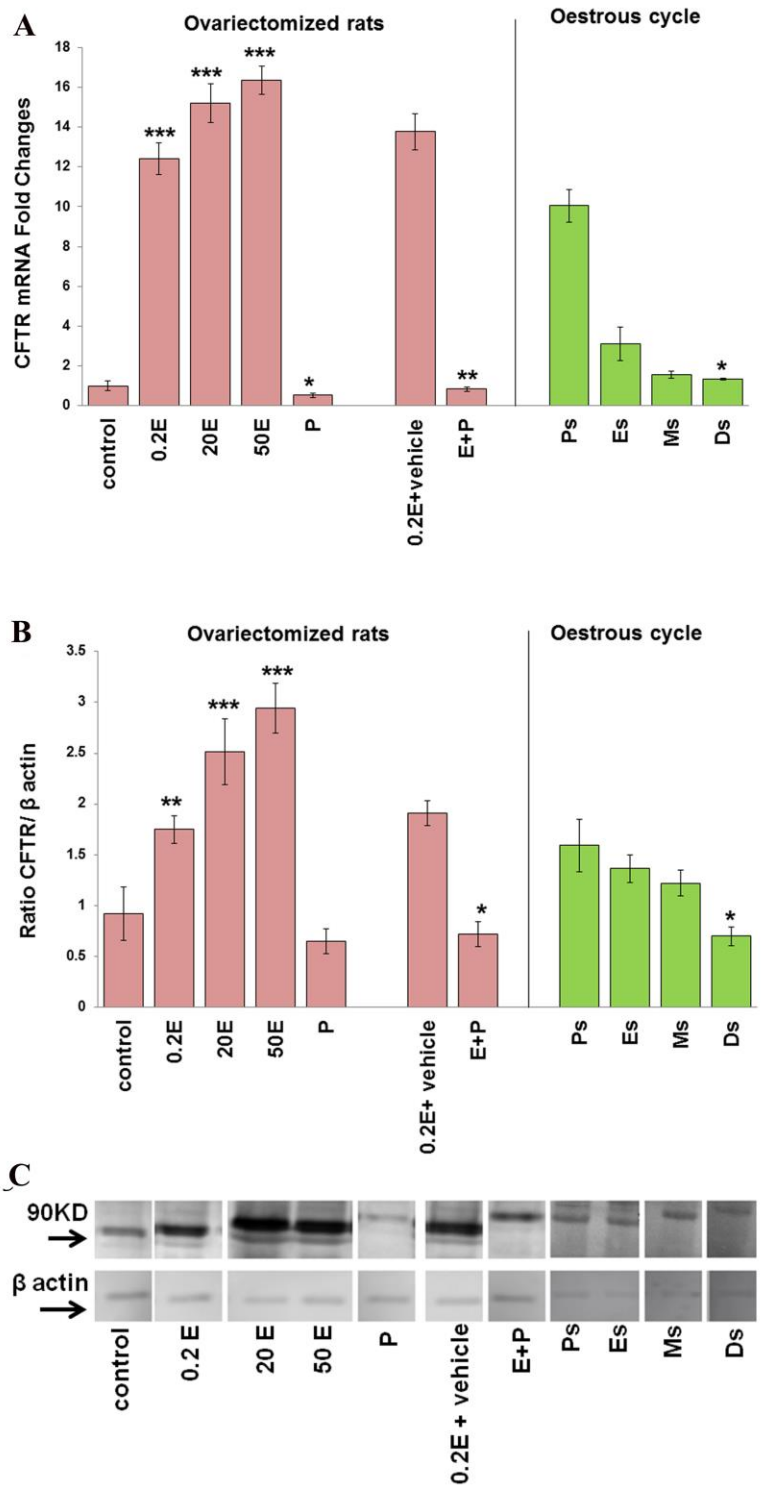



**Figure 4. 1** Real-time PCR (A) and Western blotting analysis (B) of total homogenate of the uterus; representative photo of western blots (C) of CFTR in ovariectomized rats and rats at different stages of the oestrous cycle. The photos as shown in (C) came from a same gel/blot that has been cut in order to arrange the different groups of treatment in sequence as to match the result in (A) and (B). 0.2E: 0.2µg estrogen, 20E: 20µg estrogen, 50E: 50µg estrogen, P: progesterone, E+P: 0.2µg estrogen + 4mg progesterone. 0.2E + vehicle: 0.2$\mu$g estrogen + peanut oil Ps: proestrus, Es: estrus, Ms: metestrus, Ds: diestrus. n=4 per group (3technical replicate*)* *, $P < 0.05$, **$P < 0.01$, ***$P < 0.001$, data presented as mean ±SEM, compared to control in ovariectomized rat, however E+P compared to 0.2+ vehicle and compared to Es at different stages of oestrous cycle.

### .1.3 Immunolocalization of CFTR

An increase in CFTR expression was observed in the E treated group in dose dependent manner and mainly localized at the apical membrane of the luminal epithelia. CFTR distribution could also be seen in the glandular epithelia. No staining was seen in the stroma or myometrium. Meanwhile, CFTR expression was reduced following P and E + P treatments (Figure 4.2 & Table 4.1).

The expression of CFTR was increased at estrus mainly at the apical membrane of the luminal epithelia. Enhanced expression of this protein was observed in the glandular epithelia with minimal expression in the stroma. At diestrus however, the amount of CFTR protein expression was very low compared to estrus (Figure 4.2 & Table 4.1).

**Table 4. 1** Semiquantitative of CFTR in the uterine luminal endometrium by IHC

| group | apical | basal |
|---|---|---|
| control | + | - |
| 0.2µg estrogen | ++ | - |
| 20 µg estrogen | +++ | +/- |
| 50 µg estrogen | +++ | + |
| 4mg progesterone | +/- | - |
| 0.2µg estrogen +4mg progesterone | + | - |
| 0.2µg estrogen+ vehicle | ++ | +/- |
| proestrus | ++ | + |
| estrus | +++ | + |
| metestrus | + | - |
| diestrus | + | - |

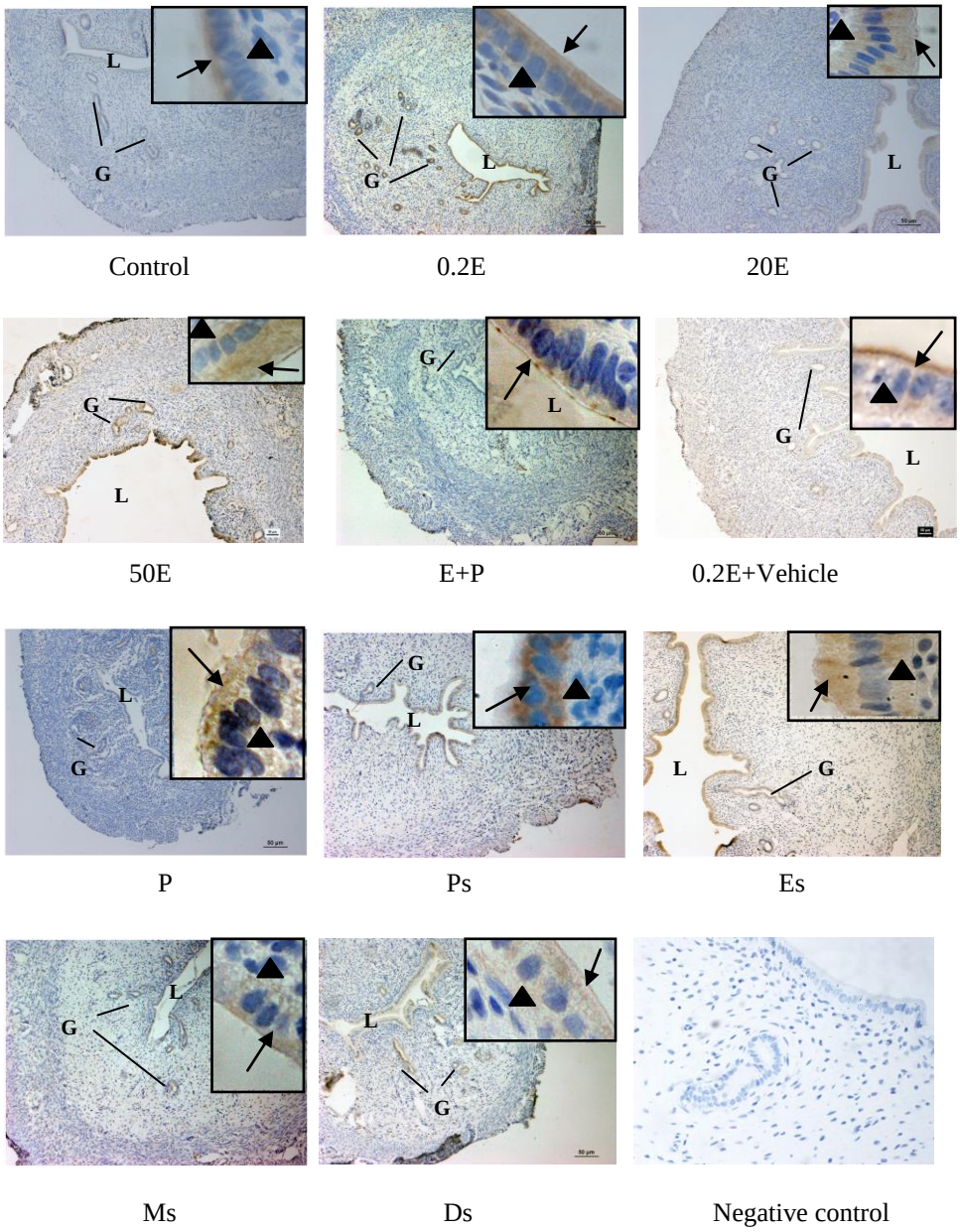



**Figure 4. 2** Immunodistribution of CFTR under the effect of exogenous and endogenous sex-steroid hormones. Magnifications 10X and 100X (in the upper right corner). 0.2E: 0.2µg estrogen, 20E: 20µg estrogen, 50E: 50µg estrogen P: 4mg progesterone, E+P: 0.2µg estrogen +4mg progesterone, 0.2E+vehicle: 0.2µgoestrogen+ peanut oil, Ps: proestrus, Es: estrus, Ms: metestrus, Ds: diestrus. IHC with goat nonspecific immunoglobolin is negative control to check the specificity of antibodies. No staining was observed in this experiment. L: lumen, G: Gland, arrow: luminal apical membrane, arrow head: basolateral membrane.

## 4.2 mRNA level, protein expression and localization of SLC26A6

### 4.2.1 SLC26A6 mRNA and protein in ovariectomized rats

In Figure 4.3.A, real-time PCR showed that the expression of SLC26A6 mRNA was under the influence of sex steroids. Under P influence, SLC26A6 mRNA level was increased relative to the control. The mRNA expression under P was lower than 0.2E. There was a dose-dependent increase in the expression of SLC26A6 mRNA under E, ranging between 1.5 and 5-fold increase following treatment with 0.2E and 50E, respectively. Treatment with E followed by P (E + P) resulted in a significant reduction in the mRNA level, which indicates that P acutely suppressed the expression of SLC26A6 mRNA induced by E. There was a significant reduction in SLC26A6 mRNA level following treatment with E + P as compared to 0.2E + vehicle.

Western blot analysis of SLC26A6 protein showed similar changes to the mRNA. P resulted in a decrease in the expression of SLC26A6 protein as compared to E. A dose-dependent increase in protein expression was observed following E treatment. A 0.15- to 0.2-fold increase in SLC26A6 protein expression was seen following treatment with 0.2E, and 50E respectively. Administration of P after three days of treatment with 0.2E did not result in a significant reduction in the expression of SLC26A6 protein as compared to 0.2E alone or E treatment followed by vehicle (a control group for E + P), (Figure 4.3.B).

### 4.2.2 SLC26A6 mRNA and protein throughout the oestrous cycle

Figure 4.3.A indicated that throughout the oestrous cycle, an increase in SLC26A6 mRNA expression by 3.2 and 3.5 fold could be seen at proestrus and estrus stages, respectively. The mRNA level was, however, reduced at metestrus and sharply declined

at diestrus. These observations indicate that SLC26A6 mRNA expression was upregulated by E, while P, at diestrus, down regulates its expression.

The highest protein expression was observed at proestrus while the lowest expression could be seen at diestrus, as can be seen in figure 4.3.B.

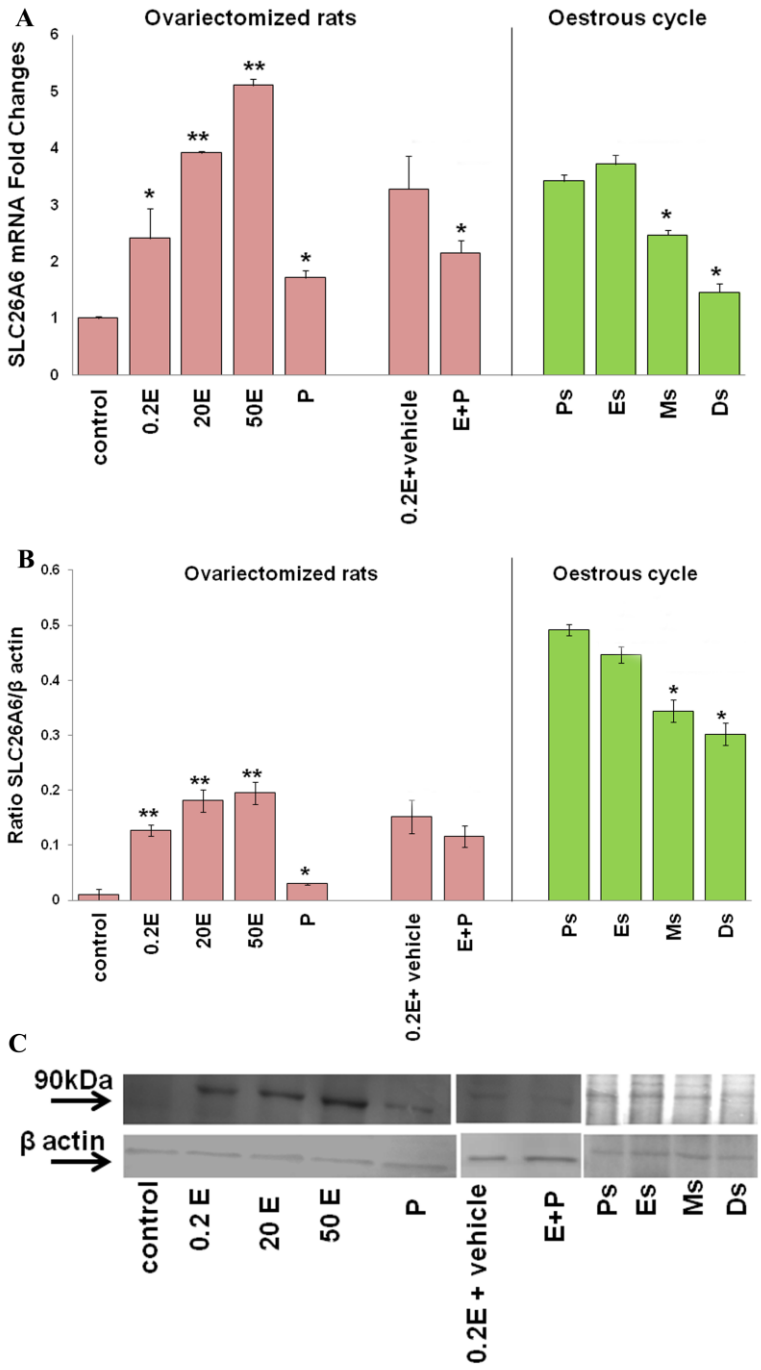



**Figure 4. 3** Real-time quantitation PCR (A) and western blotting analysis (B) of total homogenate of the uterus; representative photo of western blots (C) of SLC26A6 in ovariectomized rats and rats at different stages of the oestrous cycle. The photos as shown in (c) came from a same gel/blot that has been cut in order to arrange the different groups of treatment in sequence as to match the result in (A) and (B). 0.2E: 0.2µg estrogen, 20 E: 20µg estrogen, 50E: 50µg estrogen, P: 4mg progesterone, E+P: 0.2µg estrogen + 4mg progesterone. 0.2E + vehicle: 0.2$\mu$g estrogen + peanut oil Ps: proestrus, Es: estrus, Ms: metestrus, Ds: diestrus. n=4 *, $P < 0.05$, **$P < 0.01$, ***$P < 0.001$, data presented as mean ±SEM.

### 4.2.3 Immunolocalization of SLC26A6

The expression of SLC26A6 was increased under E effect in a dose dependent manner, with the highest staining at the apical membrane of the luminal epithelia. SLC26A6 could be seen in glandular epithelia as well as stroma following E treatment. Increased expression was also noted in the E + P group, however the intensity was lesser than in E treated group. In the P treated group however, minimal staining could be seen (Figure 4.4 & Table 4.2).

SLC26A6 expression was increased at proestrus and estrus, with a higher intensity at estrus mainly at the apical membrane of both the luminal and glandular epithelia. Minimal distribution could be seen at metestrus. At diestrus however, no staining was seen in the endometrium (Figure 4.4 & Table 4.2).

**Table 4. 2** Semiquantitative of SLC26A6 in the uterine luminal endometrium by IHC

| group | apical | basal |
|---|---|---|
| control | + | - |
| 0.2µg estrogen | ++ | + |
| 20 µg estrogen | +++ | + |
| 50 µg estrogen | +++ | + |
| 4mg progesterone | +/- | +/- |
| 0.2µg estrogen +4mg progesterone | + | - |
| 0.2µg estrogen+ vehicle | ++ | +/- |
| proestrus | ++ | + |
| estrus | +++ | + |
| metestrus | + | + |
| diestrus | + | +/- |

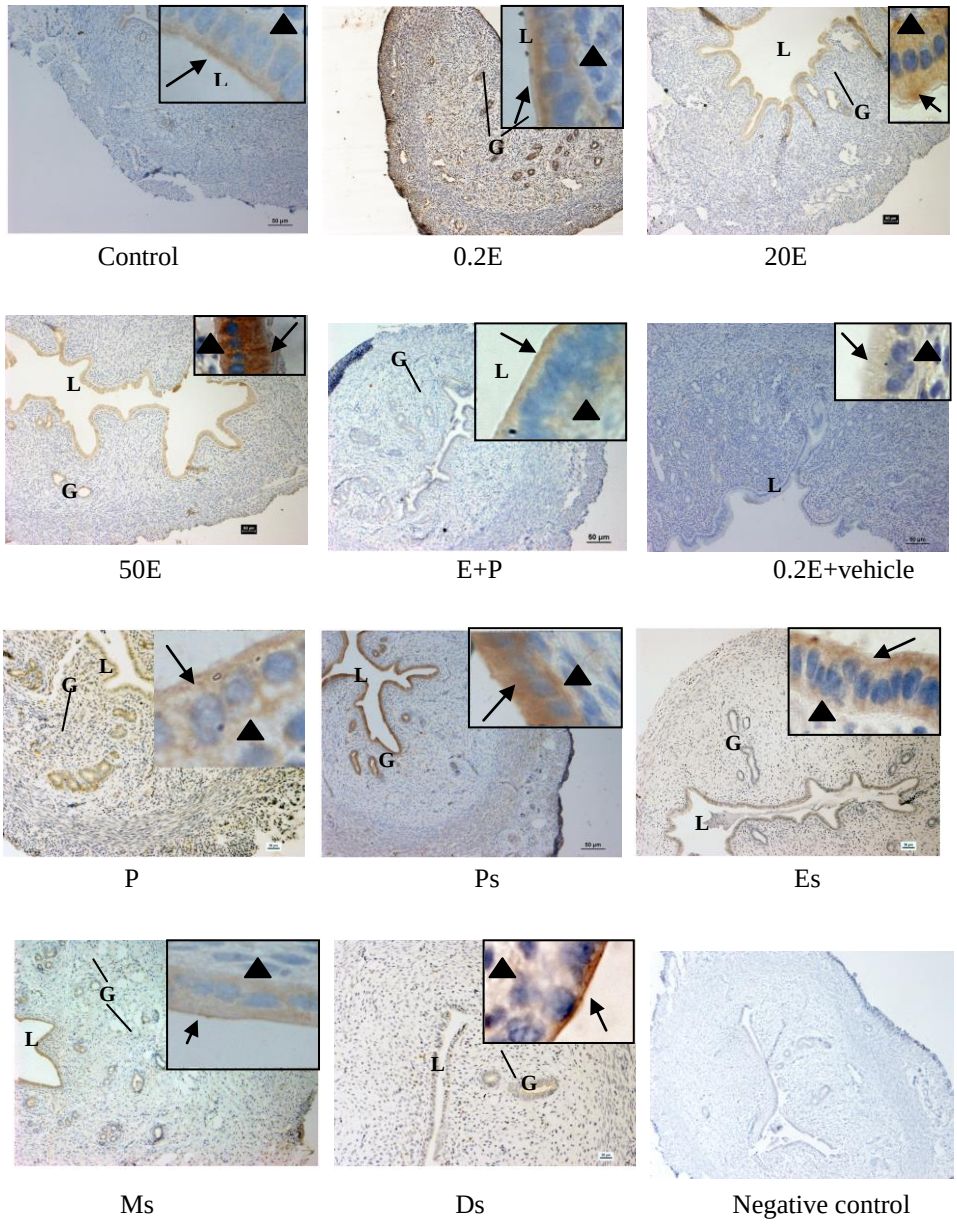



**Figure 4. 4** Immunodistribution of SLC26A6 under the effect of exogenous and endogenous sex-steroid hormones. Magnifications 10X and 100X (in the upper right corner). 0.2E: 0.2µg estrogen, 20E: 20µg estrogen, 50E: 50µg estrogen P: 4mg progesterone, E+P: 0.2µg estrogen +4mg progesterone, 0.2E+vehicle: 0.2µg estrogen+ peanut oil, Ps: proestrus, Es: estrus, Ms: metestrus, Ds: diestrus. IHC with goat nonspecific immunoglobolin is negative control to check the specificity of antibodies. No staining was observed in this experiment. L: lumen, G: Gland, arrow shows luminal apical membrane, arrow head shows basolateral membrane

## 4.3 mRNA level, protein expression and localization of NBCe1

### 4.3.1 NBCe1 mRNA and protein expression in ovariectomized rats

The qPCR assay and primary antibody that was used to evaluate SLC4A4 was for NBCe1.

Evaluating the NBCe1 mRNA level in E treated groups showed higher level of mRNA compared to control; however there was no difference among different E treatment. P and E+P treatment presented no remarkable difference with respect to control group (Figure 4.5.A).

NBCe1 showed band at 135kDa (Figure 5.4.C). NBCe1 protein expression, however revealed that E treatment stimulated NBCe1 protein expression in dose dependent manner, whereby higher NBC protein observed in 50E group compared to control rats. P decreased NBCe1 expression while E+P showed no difference in compare to control group (Figure 5.4.B). Differences between NBCe1 mRNA level and protein expression may indicated the presence of different NBCe1 variant as reported by (Liu, *et al.*, 2012).

### 4.3.2 NBC mRNA level and protein expression throughout oestrous cycle

Investigating the alteration in NBCe1 mRNA level, showed that although NBCe1 mRNA reduced from proestrus to diestrus, only reduction in diestrus was significantly vary with estrus (2.28 *vs* 1.31), Figure 4.5.A.

NBCe1 protein expression was parallel to mRNA level, whereby proestrus and estrus showed the highest NBCe1 protein, followed by significant reduction in metestrus and diestrus compared to estrus.

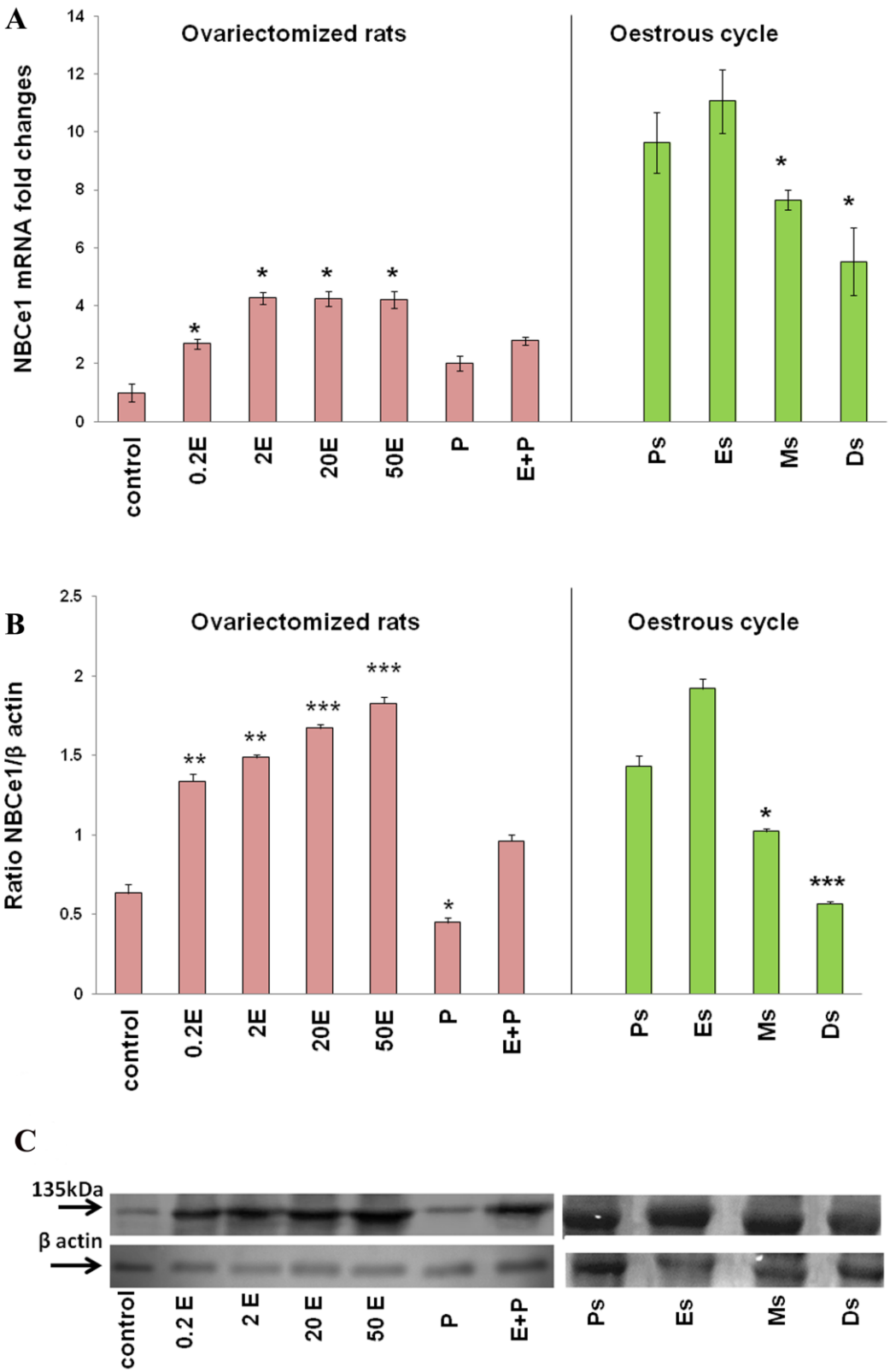



**Figure 4. 5** Gene expression analysis by qPCR (A) and western blotting analysis (B); and representative photo of western blots (C) of NBCe1 in ovariectomized rats and rats at different stages of the oestrous cycle. 0.2E: 0.2µg estrogen, 2E: 2 µg estrogen, 20 E: 20µg estrogen, 50E: 50µg estrogen, P: 4mg progesterone, E+P: 0.2 µg estrogen + 4mg progesterone. Ps: proestrus, Es: estrus, Ms: metestrus, Ds: diestrus. n=4, each has 3 technical replicate,*, $P < 0.05$, **$P < 0.01$, ***$P <$ 0.001, data presented as mean ± SEM. In ovariectomized rats, different groups were compared to control, however at oestrous cycle comparison was with Es.

### 4.3.3 Immunolocalization of NBCe1

In figure 4.6 and table 4.3 showed NBCe1 was predominantly found at the luminal and glandular epithelia following E treatment, with lack of expression in the stroma and myometrium. At higher magnification, NBCe1 could be seen at the apical and basolateral membrane of the luminal epithelia with a higher intensity in the former. NBCe1 was observed in stroma and myometrium at an increasing intensity with increasing doses of E. Following P treatment and in the E + P treated group, a marked decreased in luminal and glandular expression of NBCe1 was observed with no staining seen in the stroma and myometrium.

At proestrus and estrus bilateral expression of NBCe1 was found at the luminal epithelia. NBCe1 was seen in glandular epithelia in addition to stromal and myometrial expression during proestrus and estrus. At diestrus however, minimal staining was observed at the luminal and glandular epithelia.

**Table 4. 3** Semiquantitative of NBCe1 in the uterine luminal endometrium by IHC

| group | Apical | basal |
|---|---|---|
| control | + | - |
| 0.2µg estrogen | + | + |
| 2 µg estrogen | ++ | ++ |
| 20 µg estrogen | +++ | +++ |
| 50 µg estrogen | +++ | +++ |
| 4mg progesterone | + | - |
| 0.2µg estrogen +4mg progesterone | + | + |
| proestrus | ++ | + |
| estrus | ++ | ++ |
| metestrus | ++ | +/- |
| diestrus | + | - |

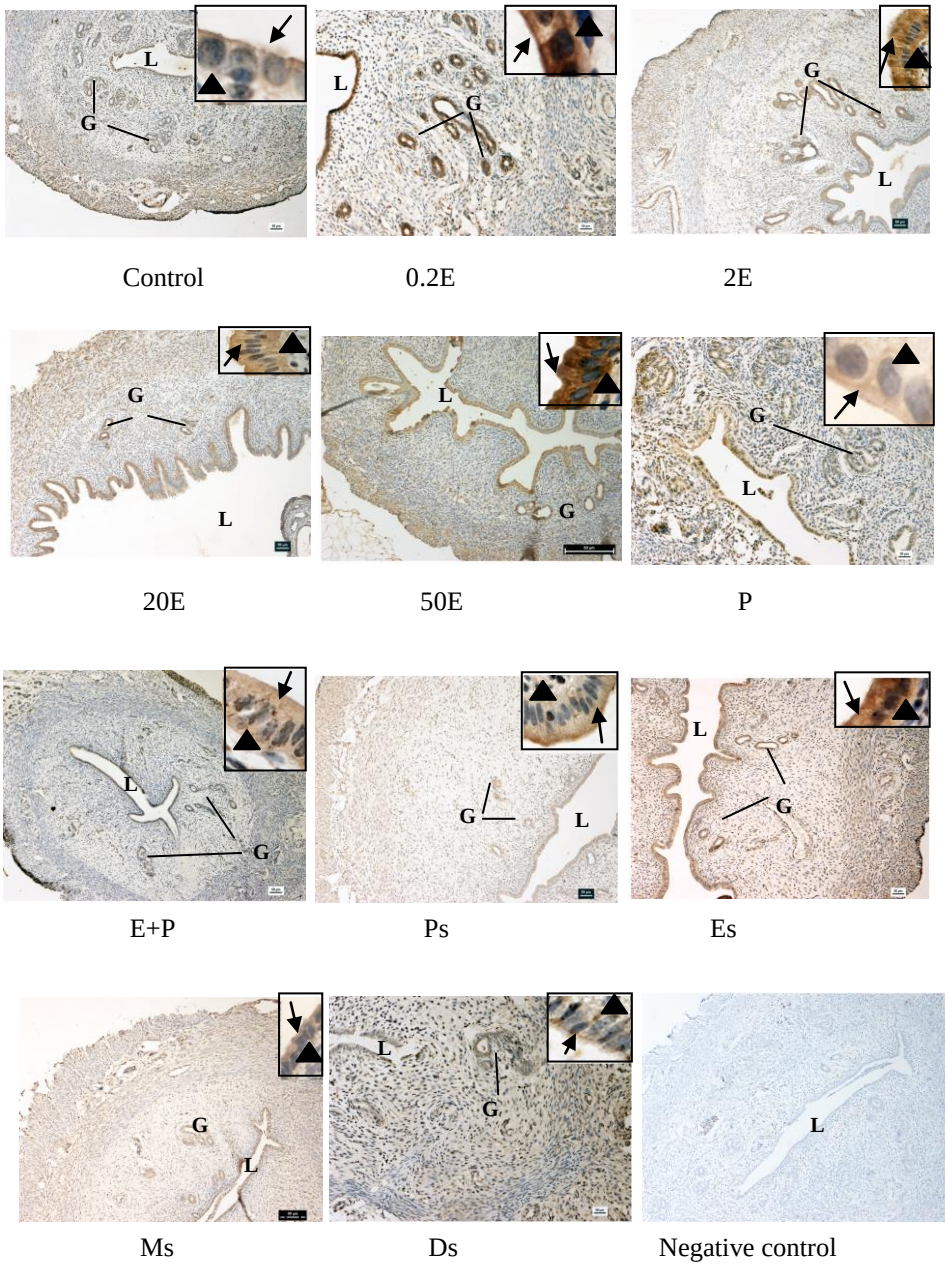



**Figure 4. 6** Immunodistribution of NBCe1 in steroid treated ovariectomized rats and at different stages of oestrous cycle. Magnifications 10X and 100X (in the upper right corner). 0.2E: 0.2µg estrogen, 2µg estrogen, 20E: 20µg estrogen, 50E: 50µg estrogen P: 4mg progesterone, E+P: 0.2µg estrogen +4mg progesterone, Ps: proestrus, Es: estrus, Ms: metestrus, Ds: diestrus. IHC with rabbit nonspecific immunoglobolin is negative control to check the specificity of antibodies. No staining was observed in this experiment. L: lumen, G: Gland, arrow shows luminal apical membrane, arrow head shows basolateral membrane.

## 4.4 mRNA level, protein expression and localization of CAII

### 4.4.1 CAII mRNA and protein expression in ovariectomized rats

Under the effect of 0.2E, CA II mRNA level was increased by 70.2 folds. Interestingly, in the 2E group there was a decrease in CAII mRNA expression (13 folds). Treatment with 20E resulted in 2 folds increase, while 4 folds increase in mRNA level of CAII was seen in 50E group. P treatment meanwhile caused in only 48 folds increase while treatment with 0.2E followed by 4 mg P led to 83 folds increase in the CAII mRNA level , figure 4.7.A.

CA II protein expression followed an almost similar pattern to the changes in mRNA level following sex-steroid treatment. The highest expression was noted following treatment with E + P (7.2 folds). While it was only 6.6 folds increase in CAII protein expression in 0.2E group. There were no significant increase in the protein expression following treatment with 2 and 20E (2.8 & 2.6-folds) as compared to the control, while treatment with 50E resulted in a 3.05 folds increase. Treatment with P led to 5.2 folds increase and was significantly higher than the control, figure 4.7.B.

### 4.4.2 CAII mRNA and protein expression throughout the oestrous cycle

Throughout the oestrous cycle, the highest increase in CAII mRNA level was noted at estrus (32 folds), followed by proestrus (24 folds) while the lowest mRNA level was observed at diestrus (6 folds). At metestrus however, there was only a 5 folds increase in the mRNA level (Figure 4.7.A).

At oestrous cycle, the highest CAII protein expression was noted at estrus (2 folds increase), while at proestrus, there was only a 1.9 fold increase. The lowest protein expression was occurred at metestrus (1.1 folds) and diestrus (1.2 fold), (Figure 4.7.B). The molecular weight of the CA II protein was detected at 29 kDa, (Figure 4.7.C).

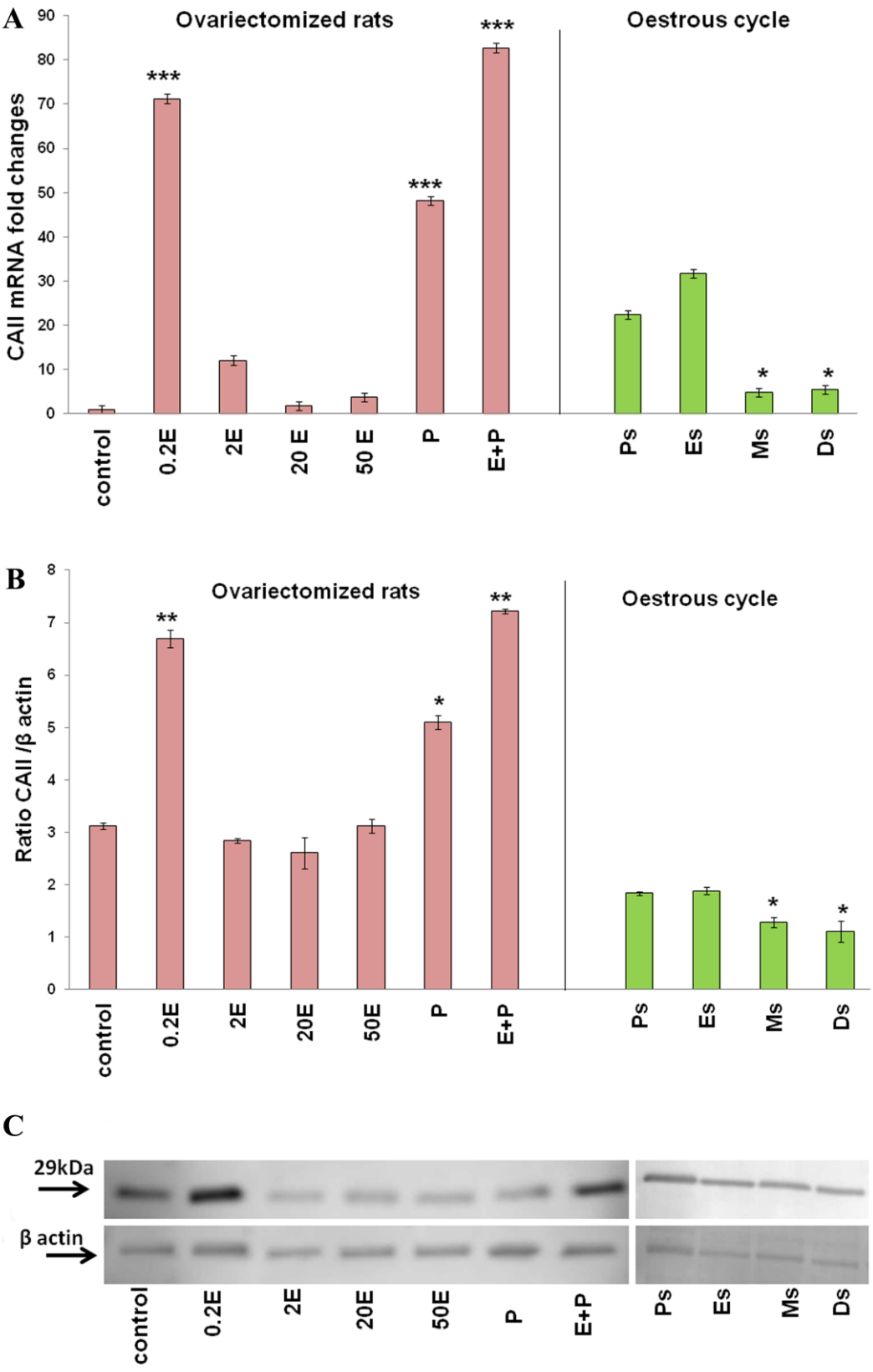



**Figure 4. 7** Analysis of mRNA level (A), protein expression (B) and representative blot (C) of CAII. 0.2E: 0.2µg estrogen, 2E: 2µg estrogen, 20 E: 20µg estrogen, 50E: 50µg estrogen, P: progesterone, E+P: 0.2µg estrogen + 4mg progesterone. Ps: proestrus, Es: estrus, Ms: metestrus, Ds: diestrus. n=4, each has 3 technical replicate, *, $P < 0.05$, **$P < 0.01$, ***$P < 0.001$, data presented as mean ± SEM. In ovariectomized rats comparison was done with control group, however at oestrus cycle the significance of the result compared with Es.

### 4.4.3 Immunolocalization of CA II

Figure 4.8 and table 4.4 showed the immunolocalization of CAII under the influence of different steroid in ovariectomized rats and throughout the oestrous cycle.

Under all treatment, CAII expression in the glandular epithelium was with much higher intensity than luminal epithelium. However under the effect of 0.2E it was more intense than other groups (Figure 4.8 and table 4.4).

Immunolocalization of CAII showed that during all stages of the oestrous cycle, CAII was expressed on the glandular epithelium (Figure 4.8) CAII was highly expressed in the glandular epithelia, however its expression in the luminal epithelia is rather low (Figure 4.8). Thus glandular magnification was presented in figure 4.8 and semi quantification of CAII in the uterine glandular epithelium by IHC has summarized in table 4.4.

**Table 4. 4**Semi quantification of CAII in the uterine glandular epithelium by IHC

| group | Glandular epithelium |
|---|---|
| control | + |
| 0.2µg estrogen | ++ |
| 2µg estrogen | + |
| 20µg estrogen | + |
| 50µg estrogen | + |
| 4mg progesterone | ++ |
| 0.2µg estrogen +4mg progesterone | ++ |
| proestrus | +++ |
| estrus | ++ |
| metestrus | + |
| diestrus | ++ |

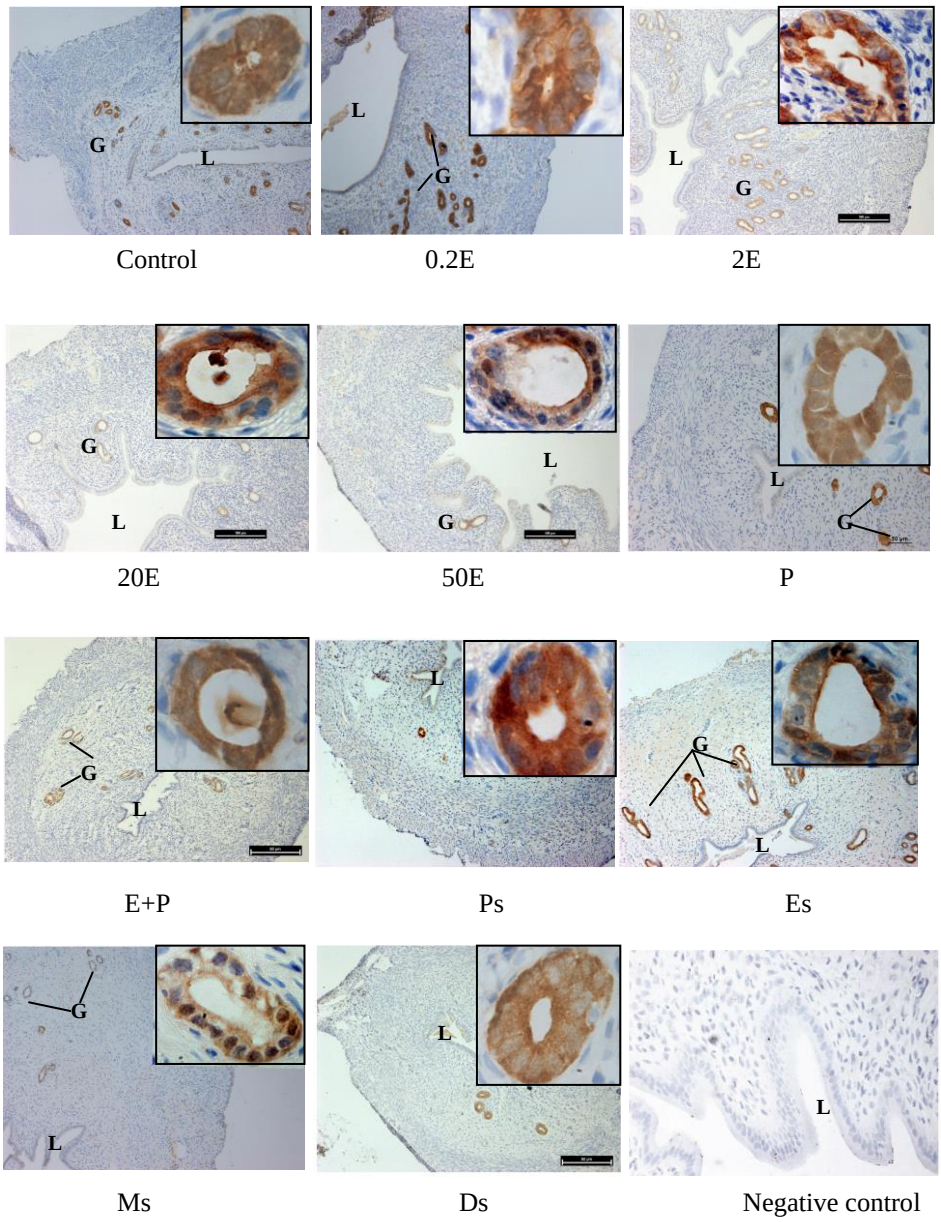



**Figure 4. 8** Immunodistribution of CAII in steroid treated ovariectomized rats and at different stages of the oestrous cycle. Magnifications 10X, whole uterus and 100X (in the upper right corner, gland). 0.2E: 0.2µg estrogen, 2µg estrogen, 20E: 20µg estrogen, 50E: 50µg estrogen P: 4mg progesterone, E+P: 0.2µg estrogen +4mg progesterone, Ps: proestrus, Es: estrus, Ms: metestrus, Ds: diestrus. IHC with mouse nonspecific immunoglobolin is negative control to check the specificity of antibodies. No staining was observed in this experiment. L: lumen, G: Gland.

## 4.5 mRNA level, protein expression and localization of CAXII

### 4.5.1 CAXII mRNA and protein evaluation in ovariectomized rats

Under the effect of 0.2E, CA XII mRNA level was increased by 10.5 folds. However treatment with 2E reduced CA XII mRNA level to 1.5 fold. Treatment with 20E resulted in 2 folds increase, while 50E resulted in 8.2 folds increase. P treatment resulted in 8.0 folds increase while exposure to 0.2E followed by 4 mg P (E+P) resulted in further increase to 12.2-folds (Fig 4.9A).

The changes in the expression of CAXII protein are almost similar to the changes in mRNA level following sex-steroid treatment. The highest increase was noted following treatment with E+P (1.9 fold) followed by 0.2E treatment (1.3 fold). There were no significant changes in the protein expression following treatment with 2 and 20E as compared to the control. Meanwhile, treatment with 50E resulted in a significant increase in CAXII protein expression (1.0 fold) as compared to the control. P treatment resulted in 1.1 fold increase which was significantly higher than the control (Fig 4.9.B). CAXII showed band at 44kDa (figure 4.9.C).

### 4.5.2 CAXII mRNA and protein expression throughout the oestrous cycle

Throughout the oestrous cycle, the highest level of CAXII mRNA was noted at estrus (15.5 folds), followed by proestrus (10 folds) while at diestrus, there was only a 3.8 folds increase in the mRNA level. At metestrus however, there was only a 7.5 folds increase in CAXII mRNA level (Figure 4.9.A).

Throughout the oestrous cycle, the highest CAXII protein expression was noted at estrus (1.65 fold), while at proestrus, there was only a 1.35 fold increase. The lowest expression was noted at metestrus (0.9 fold) followed by diestrus (0.65 fold), figure 4.9.B. The molecular weight of CA XII protein was observed at 44kDa, (Fig 4.9.C).

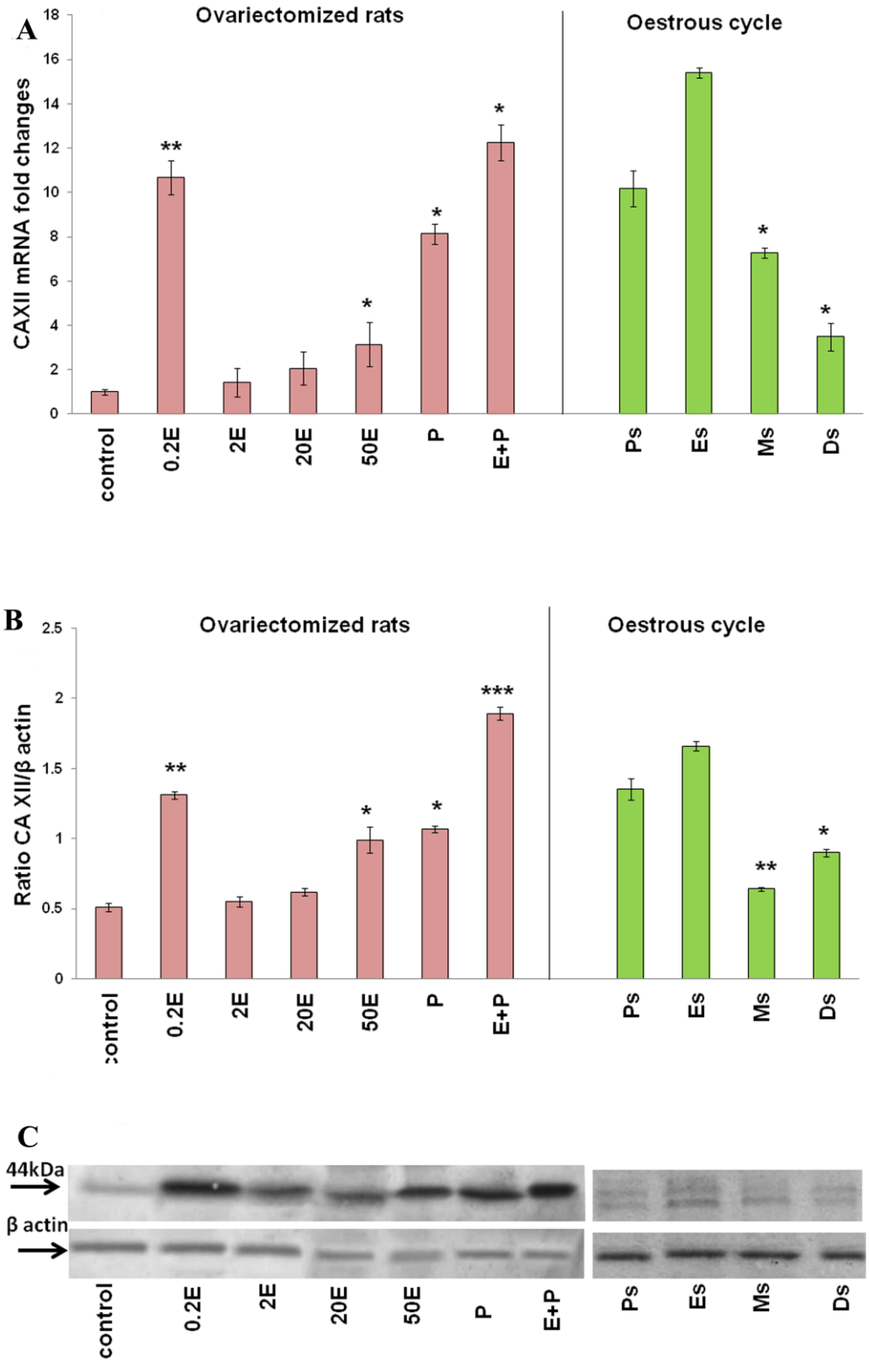



**Figure 4. 9** Effect of exogenous and endogenous steroid hormone on CAXII gene expression (A), western blotting analysis (B) and respective blot (C). 0.2E:0.2µg estrogen, 2E:2µg estrogen, 20 E:20µg estrogen, 50E: 50µg estrogen, P: 4mg progesterone, E+P: 0.2µg estrogen + 4mg progesterone. Ps: proestrus, Es: estrus, Ms: metestrus, Ds: diestrus, n=4, each has 3 technical replicate *, $P < 0.05$, **$P < 0.01$, ***$P < 0.00$, data presented as mean ± SEM. In ovariectomized rats comparison was done with control group, however at oestrus cycle the significance of the result compared with Es.

### 4.5.3 Immunolocalization of CAXII

In figure 4.10 and table 4.5, CAXII expression was the highest in the uterine luminal epithelia in all treatment groups and at all phases of the oestrous cycle. Under 0.2E, E+P, at proestrus and estrus CAXII was more intensely distributed at the basolateral as compared to the apical membrane. Under the effect of P and at diestrus, this protein was predominantly expressed at the apical membrane. Although treatment with 2E resulted in a predominant apical expression, higher E doses at 20 and 50E resulted in a predominant basolateral expression.

**Table 4. 5** Semi quantitative of CAXII in the uterine luminal endometrium by IHC

| group | apical | basal |
|---|---|---|
| control | + | - |
| 0.2µg estrogen | + | + |
| 2µg estrogen | ++ | ++ |
| 20µg estrogen | +++ | +++ |
| 50µg estrogen | +++ | +++ |
| 4mg progesterone | + | - |
| 0.2µg estrogen +4mg progesterone | + | + |
| proestrus | ++ | + |
| estrus | ++ | ++ |
| metestrus | ++ | +/- |
| diestrus | + | - |

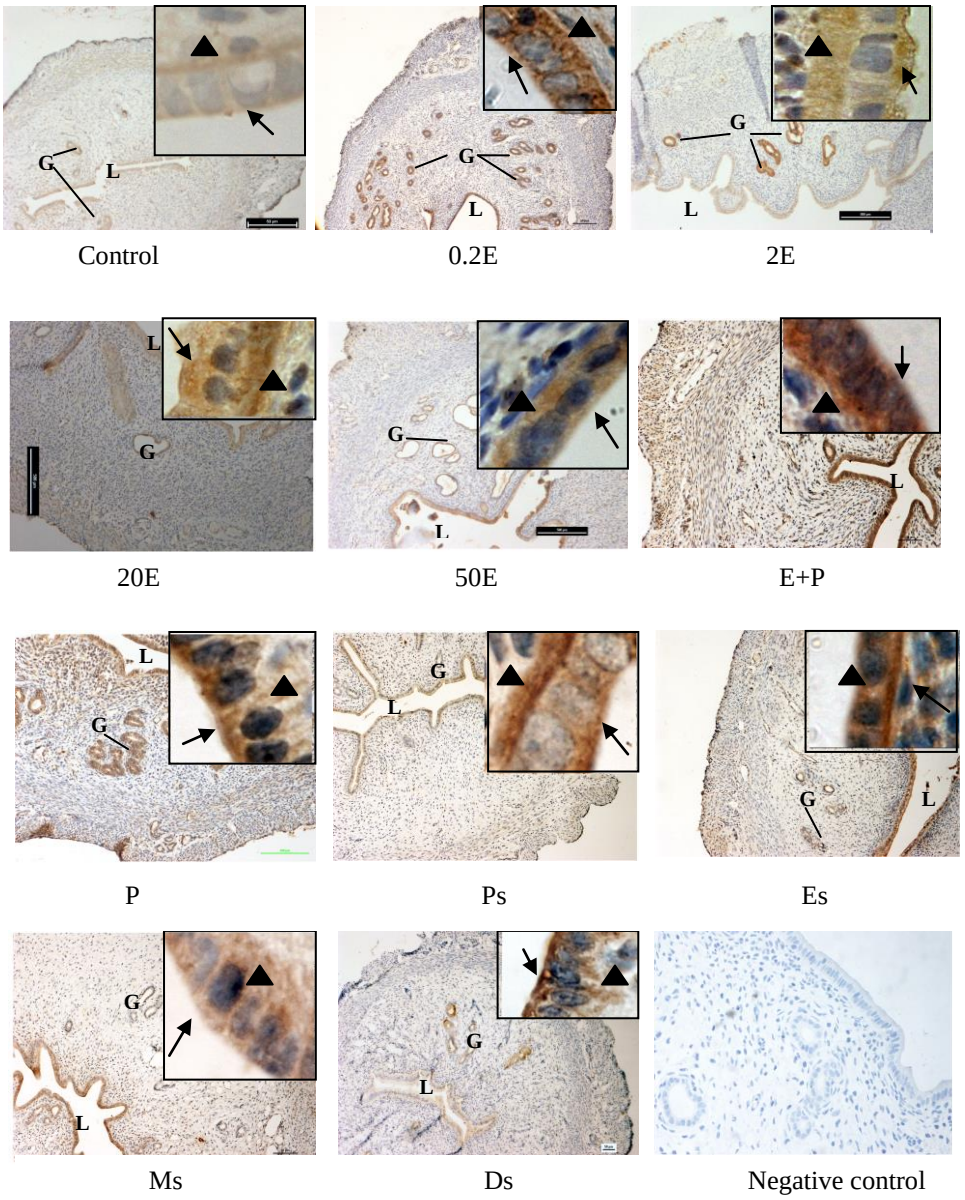



**Figure 4. 10** Immunodistribution of CAXII in steroid treated ovariectomized rats and at different stages of THE oestrous cycle. Magnifications 10X and 100X (in the upper right corner). 0.2E: 0.2µg estrogen, 2µg estrogen, 20E: 20µg estrogen, 50E: 50µg estrogen P: 4mg progesterone, E+P: 0.2µg estrogen + 4mg progesterone, Ps: proestrus, Es: estrus, Ms: metestrus, Ds: diestrus. IHC with rabbit nonspecific immunoglobolin is negative control to check the specificity of antibodies. No staining was observed in this experiment. L: lumen, G: Gland, arrow: luminal apical membrane, arrow head: basolateral membrane

## 4.6 Discussion

### 4.6.1 CFTR and SLC26A6 expression

The actual mechanism involved in the control of uterine luminal fluid volume is still a matter of debate among reproductive biologists. The first evidence regarding the role of CFTR in the regulation of in-uterine fluid volume surfaced nearly 20 years ago when Rochwerger and Buchwald (1993), using *in situ* hybridization and protein studies, reported that E stimulates expression of CFTR in uterus. Forskolin-stimulated $Cl^-$ permeability was observed at the apical membrane of the endometrial cells while *in vitro* primary cultures of rat uterine epithelial cells showed high levels of CFTR expression under E stimulation (Rochwerger, *et al.*, 1994). In contrast to E, Mularoni *et al* (1995), using a competitive RT-PCR technique, reported that CFTR mRNA level in the glandular epithelial cells of guinea-pig endometrium decreases upon treatment with E plus P as compared to the treatment with E in an *in vitro* study.

Apart from this limited observation, the actual effect of P on uterine CFTR expression is still not fully elucidated. This part of the present study was undertaken to investigate the actual effect of P treatment alone and the effect of P following exposure to E on the mRNA and protein expression of CFTR as well as SLC26A6 in the uterine tissues in a rat model.

A complimentary finding by Chan, *et al* (2002) indicates fluctuation in CFTR mRNA expression in adult female mouse reproductive tract at different phases of the oestrous cycle. *In situ* hybridization and semi-quantitative RT-PCR revealed abundant expression of CFTR mRNA in the uterine epithelium at estrus but not at other stages of the cycle. In contrast to Chan, *et al* (2002), our finding, however, indicates that CFTR mRNA expression is the highest at proestrus (Figure 4.1.A). These differences in the time of CFTR mRNA expression could probably be related to species difference, in which Chan, *et a*l (2002) conducted their study in mice in contrast to our rat model.

Yang, *et al* (2004) studied CFTR expression in pregnant mice by RT-PCR, which showed a maximum CFTR expression at day 3 after-mating predominantly in the stromal cells. As can be seen from figure 4.2 CFTR is highly expressed at the apical membrane of rat endometrium and was up-regulated by E. Functional study showed that CFTR is involved in uterine $Cl^-$ (Figure 6.3.B) and fluid secretion (Figure 6.2.B) under E stimulation. Meanwhile, Nobuzane, *et al* (2008) reported that treatment with raloxifene analogue induced CFTR expression in a study using RT-PCR. An exact determination of the amount of mRNA is not possible by a traditional PCR, therefore real time PCR (as used in this study) is the best alternative that offers an accurate quantification of mRNA expression as little as a twofold increase (Ben Abda, *et al.*, 2011). Results indicate that CFTR mRNA expression is highly increased following exogenous E administration and under a high level of endogenous E (Figure 4.1.A); therefore, CFTR could be the major anion channel expressed which may participate in the observed accumulation of uterine fluid under E dominance.

In addition, the dose- dependent effect of E on CFTR mRNA expression could provide a fundamental basis for the observed excessive fluid accumulation under hyperestrogenic condition such as following consumption of oral contraceptive pills (Salleh, *et al.*, 2005). To the best of our knowledge, this is the first report on the acute suppression by P on CFTR expression *in vivo* in steroid replaced ovariectomized rats as well as under a high endogenous E level (Fig 4.1), thus supporting the previous *in vitro* observation by Mularoni et al (1995).

Progesterone effect on CFTR expression could be mediated via genomic pathway possibly via CFTR gene suppression, apart from an indirect inhibition of CFTR activities via the activation of ENaC as ENaC has been shown to be a negative regulator of CFTR (Chan, *et al.*, 2001). This study thus provides novel information that P is responsible for suppression of CFTR expression *in-vivo,* which may explain the

diminished fluid secretion under a P-mediated effect. This would complement ENaC-mediated fluid imbibition, contributing to fluid loss that initiates uterine closure. Apart from being a $Cl^-$ channel, CFTR has also been reported to be involved in $HCO_3^-$ secretion.

E stimulation of $HCO_3^-$ secretion has also been shown to occur in the duodenum via CFTR and $Cl^-/HCO_3^-$ exchanger (Smith, *et al.*, 2008), which is responsible for the lower prevalence of duodenal ulcer in premenopausal women (Tuo, *et al.*, 2011). In addition to E, genistein, a phytoestrogen, has also been reported to increase duodenal $HCO_3^-$ secretion in a dose-dependent manner through stimulation of CFTR (Tuo, *et al.*, 2009). These observations support the notion that CFTR is the main $HCO_3^-$ extruder under E effect.

The involvement of SLC26A6 in mediating uterine $HCO_3^-$ secretion has received a lot of attention lately. The important role of this transporter in the uterus has been overlooked. While He, *et al* (2010) has examined SLC26A6 protein expression in mice uteri which indicates a maximum expression of this protein under E dominance; its mRNA expression is, however, unknown. We have therefore provided novel information on the E-induced up regulation and P-induced down regulation of uterine SLC26A6 mRNA expression *in vivo* (Figure 4.3.A). In addition, we have also documented for the first time that SLC26A6 mRNA exhibits cyclical changes throughout the oestrous cycle (Figure 4.3), in which its expression is increased at proestrus and estrus and decreased at diestrus in apical membrane (Figure 4.4). We postulate that the increase in SLC26A6 expression under E influence may contribute to the alkalinity of uterine fluid via contributing to $HCO_3^-$ secretion. The stoichiometry of 1 $Cl^-$ to 2 $HCO_3^-$ further assists in luminal $HCO_3^-$ accumulation in exchange with $Cl^-$, which needs to be recycled following its secretion through the CFTR. $Cl^-$ recycling may prevent excessive fluid secretion under E stimulation, which perhaps plays a role in a

negative feedback control of uterine luminal fluid secretion. Progesterone down-regulation of SLC26A6 resulted in a diminished $HCO_3^-$ secretion and together with an increased $H^+$ secretion via the NHE1 (Salleh, *et al.*, 2011) initiates fluid loss from the uterine lumen. This would therefore complement ENaC in contributing to uterine fluid loss under progesterone effect in order to initiate the process leading to "uterine closure," sandwiching the embryo between the two opposing uterine walls. A precise regulation in these proteins and mRNAs expression would contribute to an accurate control of the uterine fluid volume and pH that are required for a successful implantation.

### 4.6.2 NBCe1 expression

Our result showed the changes in NBCe1 protein expression at different phases of the oestrous cycle which indicate that NBCe1 mRNA and protein expression was up-regulated by E and at estrus and was down-regulated by P and at diestrus. There were several evidence on the presence of NBCe1 in the female reproductive tissue in which Liu, *et al* (2011) has detected two novel NBCe1 splice variants in mature mice uterus, while a functional study by Wang et al (2002) has document the presence of $Na^+$ dependent $HCO_3^-$ transport at the basolateral membrane of the endometrial epithelia. Hormonal and drugs influence on uterine $HCO_3^-$ secretion has been reported whereby adrenaline (Fong, *et al.*, 1998), prostaglandin-$E_2$ and forskolin (Fong & Chan, 1998) stimulate the endometrial $HCO_3^-$ secretion. E has been shown to stimulate the forskolin-induced $HCO_3^-$ secretion in the endometrial cells in *in-vitro* study which was inhibited by DIDS, a non-specific anion exchanger inhibitor (He, *et al.*, 2010) .

The E-induced increase in uterine NBCe1 mRNA and protein expression (Figure 4.5.A) as observed in this study indicates the involvement of this protein in E-stimulated uterine $HCO_3^-$ secretion. The findings also indicate that under the effect of E, NBCe1 protein was distributed at both the basolateral and apical membrane of the luminal and

glandular epithelia (Figure 4.6). This finding is in contrast with the previous report by Wang, *et al* (2002) in which NBC was believed to be distributed only at the basolateral membrane of the endometrial epithelia by application of short circuit current. In most secretory epithelia including the pancreatic duct (Satoh, *et al.*, 2003) and gastrointestinal tract (Bucking & Wood, 2012; Seidler, *et al.*, 2001), NBCe1 is localized at the basolateral membrane where it participates in the $HCO_3^-$ influx together with $Na^+$ (Abuladze, *et al.*, 1998). However, in the salivary gland (Roussa, 2011), cornea (Diecke, *et al.*, 2004) and epididymis (Jensen, *et al.*, 1999a), the apical NBCe1 is involved in intraluminal $HCO_3^-$ secretion. Meanwhile, in the salivary ductal epithelia, luminal NBCe1-A with a stoichiometry of $1Na^+$: 3 $HCO_3^-$ acting together with the basolateral NBCe1-B with a stoichiometry of 1 $Na^+$:2 $HCO_3^-$ resulting in the luminal $HCO_3^-$ efflux and transepithelial $HCO_3^-$ transport (Roussa, 2011). In view of this, it is possible that different NBCe1 variants (NBCe-1 A, B, C and D) which varies in the N and C-terminus (Boron, *et al.*, 2009) are expressed in the endometrial epithelial cells that participate in $HCO_3^-$ secretion. The expression of different NBCe1 splice variants in the uterus has been previously reported in the immature mice (Liu, *et al.*, 2011).

$HCO_3^-$ concentration in the fluid along the female reproductive tract has been reported to fluctuate throughout the menstrual cycle (Chan, *et al.*, 2009). In the oviductal fluid, the $HCO_3^-$ concentration was found to be approximately 35 mM during the follicular phase; peaking-up to 90 mM at around the time of ovulation (Maas, *et al.*, 1968; Maas, *et al.*, 1977) in rhesus monkey (Macaca mulatta) and 40mM/l in rat under the influence of E (Figure 6.3.A& 6.6.A) . In order to sustain a high intraluminal $HCO_3^-$ secretion, concerted actions of multiple $HCO_3^-$ transporters are required which include the apical CFTR, $Cl^-/HCO_3^-$ exchanger (SLC26A6) and NBC as well as the basolateral NBC. These could be similar to the mechanism underlying $HCO_3^-$ secretion in the pancreas under secretin stimulation. In pancreas, basolateral NBCe1 (Marino, *et al.*, 1999) and

apical CFTR and $Cl^-$/ $HCO_3^-$ exchanger (SLC26A6) (Ishiguro, *et al.*, 2007) as well as apical NBCe1 (Thevenod, *et al.*, 1999) are involved to sustain the high intraluminal $HCO_3^-$ concentration at 140 mmol/l (Hug, *et al.*, 2003). Meanwhile, a more established mechanism involving the apical and basolateral NBC has been reported to mediate $HCO_3^-$ secretion in the cornea. NBC was found at the basolateral and apical membrane of the bovine and rabbit's corneal endothelial cells. $HCO_3^-$ influx across the basolateral membrane of this cell has been reported to occur via the electrogenic NBCe1 with a stoichiometry of 2 $HCO_3^-$: 1 $Na^+$, while $HCO_3^-$ efflux across the apical membrane involved the NBCe1 isoform with a stoichiometry of 3 $HCO_3^-$: 1 $Na^+$ (Diecke, *et al.*, 2004).

In addition to extruding $HCO_3^-$, the apical NBCe1 may also participate in the luminal $Na^+$ secretion. NBC could be involved in intraluminal $Na^+$ secretion under E in addition to the movement of $Na^+$ across a 'leaky' tight junction as has been reported in the pancreas (Marino, *et al.*, 1999) and endometrium (Lindsay & Murphy, 2006). Up-to-date, there were no reports on the apical NBC involvement in $Na^+$ secretion. Another NBC isoform, NBC3 has been reported to be expressed at the apical membrane of the narrow cells in the cauda epididymidis and light (McMurtrie, *et al.* 2004) cells of the corpus and caput epididymidis which has been proposed to participate in the luminal $Na^+$ secretion (Pushkin, *et al.*, 2000). Our finding also indicates that following treatment with high E (20E & 50E), at estrus and proestrus, NBCe1 could be seen in the stroma and myometrium. Although NBC has been reported to be expressed in the cardiac (Yamamoto, *et al.*, 2007) and skeletal muscles (Kristensen, *et al.*, 2004), its smooth muscle expression is unknown. The exact role of stromal and myometrial NBCe1 expression is unknown; although it may likely involve in pHi homeostasis.

Under the influence of P however, minimal expression of NBCe1 mRNA and protein was noted, especially in the luminal and glandular epithelia (Figure 4.6). We concluded

that under the effect of P and at diestrus, down-regulation of NBCe1 occur which resulted in lower uterine fluid pH (Figure 6.2). In addition, P was also found to up-regulate the expression of sodium proton exchanger isoform-1 (NHE1), figure 5.1.B, which causes an increase in $H^+$ secretion. A reduction in $HCO_3^-$ secretion and an increase in $H^+$ secretion under P effect which were induced by P (Figure 6.1.A & 6.3.A) will result in uterine luminal fluid acidification under the P influence (Salleh, *et al.*, 2011). In conclusion, the changes in uterine NBCe1 expression under different sex-steroid effect and at different phases of the oestrous cycle provide a molecular basis underlying fluctuation in the uterine fluid pH observed under these conditions. This will therefore enhance our understanding on the control of $HCO_3^-$ secretion and the uterine fluid pH which is essential for successful reproduction as alteration in these parameters will affect sperm transport and capacitation, fertilization as well as embryo transport and implantation.

### 4.6.3 CA expression

The expression of CA II and XII isoforms has been reported in the humans (Karhumaa, *et al*., 2000), rats (Ge & Spicer, 1988) and mice (Ge & Spicer, 1988; Hynninen, *et al*., 2004; Karhumaa, *et al*., 2000) endometrium. Currently, limited information in regard to the effect of sex-steroids on protein and mRNA expression of these two isoforms has been reported in the uterus, in particular in rodents. As the kidney CAXII expression has been reported to exhibit species-specific differences (Kyllonen, *et al*., 2003; Parkkila, 2000), the changes in the uterine expression of CAII and CAXII isoforms under different sex-steroid effect and at different phases of the oestrous cycle in rats was examined to further understand their role in rodent's uterine fluid pH regulation.

In this study, the changes in CAII and XII expression throughout the oestrous cycle were parallel to the changes in their expression following treatment with 0.2E, P (Figure 4.7& 4.9); which were considered to be at physiological doses (Salleh, *et al.*, 2005). An

increase in CAII expression following treatment with 0.2E was consistent with an increase in its level at estrus. At this stage of the oestrous cycle, E was the main circulating sex-steroid. An enhance in the expression of CAII could be due to an increase in the development of the endometrial glands as this isoenzyme has been reported to be involved in regulating glands development in sheep (Hu & Spencer, 2005). In addition, the observed increase in CAII expression during this oestrous phase could also be related to higher endometrial angiogenesis as this isoenzyme expression has been reported in the endometrial blood vessels (Hynninen, *et al.*, 2004) as well as in the erythtrocytes (Chiang, *et al.*, 2001). CAII was neither expressed in the stroma nor in the myometrium under these conditions (Figure 4.8). These observed E effects on uterine CAII expression has been shown to be mediated via the ER (He, *et al.*, 2010).

This finding indicates that CAII was present at higher amount in the glands (Figure 4.8) following 0.2E treatment and at estrus (Figure 4.7) further support its proposed function. Intracellular CAII is involved in generating $HCO_3^-$ from $CO_2$ and $H_2O$, which was then expelled into the lumen via the apically located $HCO_3^-$ transporters including CFTR (Chan, *et al.*, 2002; Salleh, *et al.*, 2005) and (Figure 4.1&4.2) and SLC26A6 (Figure 4.3&4.4). CAII was found to interact with SLC26A6 (Vince, *et al.*, 2000; Vince & Reithmeier, 1998) which will further enhance luminal $HCO_3^-$ secretion. In parallel, concomitant $H^+$ production will be extruded via the basolateral NHEs; although interactions between CA and these transporters have yet to be documented.

It was observed that the expression of CAII was also increased following P treatment and at diestrus (Figure 4.7), which suggest its participation in the reduction in uterine fluid pH under these conditions (Figure 6.1.A) and (Salleh, *et al.*, 2001). This increase in CAII expression was supported by an increase in the activity of this enzyme under P effect (Lutwak-Mann, 1955; Nicholls & Board, 1967). In addition to generating $H^+$, CA could also be involved in $H^+$ secretion at the apical membrane. CAII was reported to

enhance the activity of sodium-proton exchanger-1 (NHE-1) (Li, *et al.*, 2002), which expression was increased at the apical membrane of the luminal epithelia under P influence (Figure 5.1). CAII and NHE-1 have been proposed to form a transport metabolon which helps in driving luminal $H^+$ secretion (Purkerson & Schwartz, 2007). This decrease in pH of the uterine fluid was proposed to bring the blastocyst close to the receptive endometrium to initiate the attachment phase of implantation (Song, *et al.*, 2007). From the findings, it was noted that high doses of E (2, 20 &50E) exert a strong inhibition on CAII expression. A similar finding was reported in the uterus in rats in which CAII expression was reduced following administration of 4 μg/kg/day E (Caldarelli, *et al.*, 2005). The significant of these effects were unknown; however it may explain the contraceptive effect following intake of E-only pills as well as infertility as observed in certain pathological conditions associated with a high E level.

Meanwhile, the expression of membrane-bound CAXII was found to increase following treatment with 0.2E and at estrus. We speculate that the observed increase in this isoenzyme expression could be related to the rapid growth of the endometrium as featured by glands and blood vessels proliferation. CAXII has been associated with tumor growth and progression (Hsieh, *et al.*, 2010). In addition, the expression of this isoenzyme was also found to be increased under hypoxic condition (Chiche, *et al.*, 2009; Doyen, *et al.*, 2013). These situations could be relevant to the uterus in which rapid tissue growth and proliferation may exceed growth of the blood vessels an early stage of the cycle, thus creating a partial hypoxic condition (Deligdisch, 2000). In view that hypoxia stimulates CAXII expression and activity (Maybin, *et al.*, 2011a; Maybin, *et al.*, 2011b), therefore under this situation, an increase in endometrial expression of this isoenzyme may occur.

Meanwhile, under P effect and at diestrus, CAXII was high (Figure 4.9) and distributed mainly at the apical membrane (Figure 4.10). The apical distribution of this isoenzyme

was thought to be involved in uterine fluid acidification under P influence (Figure 6.1.A). During this stage, apical CAXII catalyzes the conversion of excreted $H^+$ and pre-existing $HCO_3^-$ into $CO_2$ and $H_2O$. $CO_2$ will then diffuse into the cell for further generation of $H^+$ and $HCO_3^-$. Intracellular $HCO_3^-$ will be expelled into the plasma via an unidentified $HCO_3^-$ transporter at the basolateral membrane in order to sustain the intracellular pH homeostasis. As a consequence of down-regulation of the apical $HCO_3^-$ transporters by P (Figure 4.1,4.3&4.5) luminal $HCO_3^-$ secretion diminishes, thus further reduce the pH of the uterine fluid as a result of a net increase in $H^+$ secretion.

Treatment with E plus P (E+P) has also resulted in an increase in CAXII expression. This may support the contribution of CA in change of uterine fluid pH under both E and P effect (figure 6.2.A &6.5.A)

# Chapter 5

## Result & Discussion

# NHE ISOFORMS

## 5.1 Analysis of NHE1, NHE2 and NHE4 mRNA and protein expression

### 5.1.1 NHE1 mRNA level and protein expression in ovariectomized rats

In figure 5.1A, NHE1 mRNA level was the highest following P treatment (4.5 fold increase). There was dose-dependent increase in mRNA level with increasing doses of E (2.8 to 3.5 fold increase in higher E doses: 0.2 – 50E). Treatment with E followed by P resulted in a significant inhibition of the mRNA level to only about 1.95 fold, which was more than two times lesser than in the P treated group.

In figure 5.1.B, NHE1 protein expression was significantly increased following treatment with P (1.7 fold increased). E treatment resulted in a dose-dependent increase in NHE1 protein expression (0.6 – 0.9 fold). In E + P group, the amount of protein expressed was significantly lower than in the P treated group. The molecular weight for NHE1 is 100kDa (Figure 5.1.C)

### 5.1.2 NHE1 mRNA level and protein expression at oestrous cycle

Throughout the oestrous cycle, NHE1 mRNA level was the highest at diestrus (5.9 fold increase), followed by proestrus and estrus (3.4 and 2.9 fold increase respectively). These findings suggested that NHE1 protein expression was up-regulated under P dominance. Thus the highest amount of NHE1 protein observed at diestrus whereas the lowest expression was at metestrus (1.6 fold) (Figure 5.1).

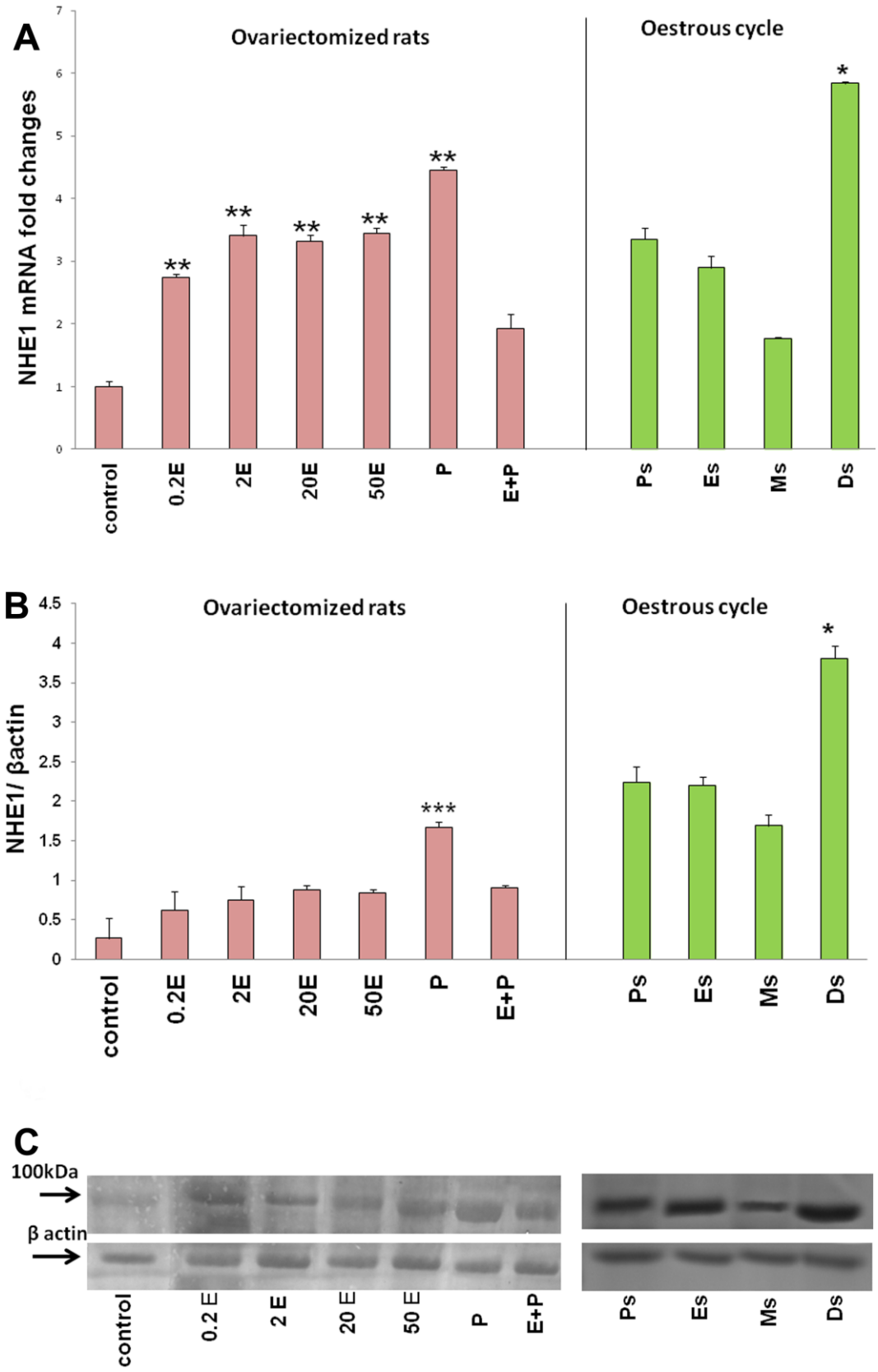



**Figure 5. 1** Comparison of mRNA level (A), protein expression analysis (B) and related blot (C) of NHE1 under the influence of steroid hormone. * compared to control group in ovariectomized rats and compared to estrus at different stages of the oestrous cycle. 0.2E: 0.2µg estrogen, 2E: 2µg estrogen, 20 E: 20µg estrogen, 50E: 50µg estrogen, P: 4mg progesterone, E+P: 0.2µg estrogen + 4 mg progesterone. Ps: proestrus, Es: estrus, Ms: metestrus, Ds: diestrus. n=4, *, $P < 0.05$, **$P < 0.01$, ***$P < 0.001$, data presented as mean ± SEM. In ovariectomized groups comparison was done with control group however at oestrous cycle comparison was done with ES.

### 5.1.3 NHE1 localization

In figure 5.2 and table 5.1, IHC results indicate that NHE1 protein was distributed mainly at the apical membrane of the luminal and glandular epithelia under steroid treatment. Minimal staining could be seen at the apical membrane in 0.2 and 2E groups, and a moderate staining predominantly at the apical membrane was observed in 20E. In the 50E group however, intense staining could be seen. In contrast to 0.2E, P resulted in intense expression of NHE1 predominantly at the apical membrane of the luminal and glandular epithelia. In E+P group, no staining was observed.

Meanwhile, throughout the oestrous cycle, NHE1 is minimally expressed at proestrus and estrus mainly at the apical membrane. At metestrus, a moderate staining could be seen at the apical membrane while an intense staining predominantly apical was observed at diestrus.

**Table 5. 1** Semi quantification of NHE1 in the uterine luminal endometrium by IHC

| group | apical | basal |
|---|---|---|
| control | - | -/+ |
| 0.2µg estrogen | - | -/+ |
| 2µg estrogen | - | + |
| 20µg estrogen | ++ | + |
| 50µg estrogen | +++ | + |
| 4mg progesterone | +++ | + |
| 0.2µg estrogen +4 mg progesterone | - | - |
| proestrus | -/+ | - |
| estrus | -/+ | - |
| metestrus | -/+ | - |
| diestrus | +++ | + |

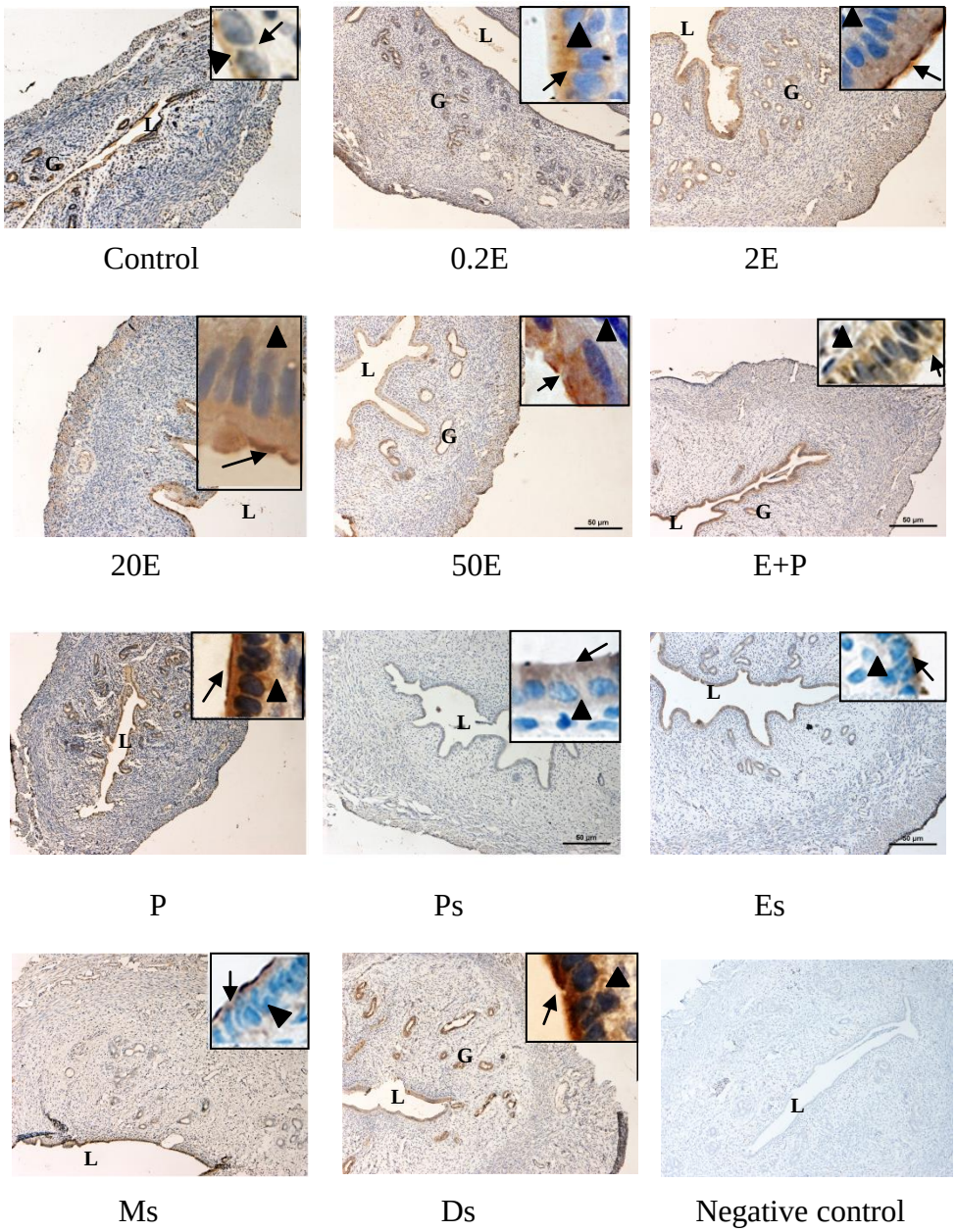



**Figure 5. 2** Immunodistribution of NHE1 in steroid treated ovariectomized rats and at different stages of oestrous cycle. Magnifications 10X and 100X (in the upper right corner). 0.2E: 0.2µg estrogen, 2µg estrogen, 20E: 20µg estrogen, 50E: 50µg estrogen P: 4mg progesterone, E+P: 0.2µg estrogen + 4mg progesterone, Ps: proestrus, Es: estrus, Ms: metestrus, Ds: diestrus. IHC with goat nonspecific immunoglobolin is negative control to check the specificity of antibodies. No staining was observed in this experiment. L: lumen, G: Gland, arrow shows luminal apical membrane, arrow head shows basolateral membrane

## 5.2 NHE2 expression

### 5.2.1 NHE2 mRNA level and protein expression in ovariectomized rats

In figure 5.3A, NHE2 mRNA expression was the highest in the group receiving 0.2E in which there was a 2.3 fold increase in mRNA level. Treatment with increasing doses of E resulted in a reduction in mRNA level (from 2.05 to 1.2 fold with 2E and 50E respectively). P treatment resulted in a low level of NHE2 mRNA. Treatment with E followed by P (E+P) did not change mRNA level of NHE2 in respect to control group.

NHE2 protein was the highest in the 0.2E group. Increasing E doses resulted in a parallel decrease in the amount of NHE2 protein expressed. P resulted in a significantly low expression (0.45 fold) as compared to 0.2E. Treatment with E + P however causes an increase in the protein amount (Figure 5.3.B).

### 5.2.2 NHE2 mRNA level and protein expression at oestrous cycle

Changes in NHE2 mRNA level throughout the oestrous cycle were consistent with its changes following different steroid treatment. At proestrus, NHE2 mRNA level was the highest with a 3.2 fold increase coincide with a high level of endogenous E, while at estrus; there was a 2.6 fold increase. The lowest mRNA level was noted at diestrus (2.1 fold), when the circulating P level was high (Figure 5.3.A), however these changes in mRNA level are not significant.

Throughout the oestrous cycle however, NHE2 protein was expressed the highest at estrus stage (2.75) and the lowest at diestrus stage (0.95). This is consistent with a high endogenous E at estrus and high plasma P at diestrus (Figure 5.3.B). The molecular weight for NHE2 is 75 kDa (Figure 5.3.C).

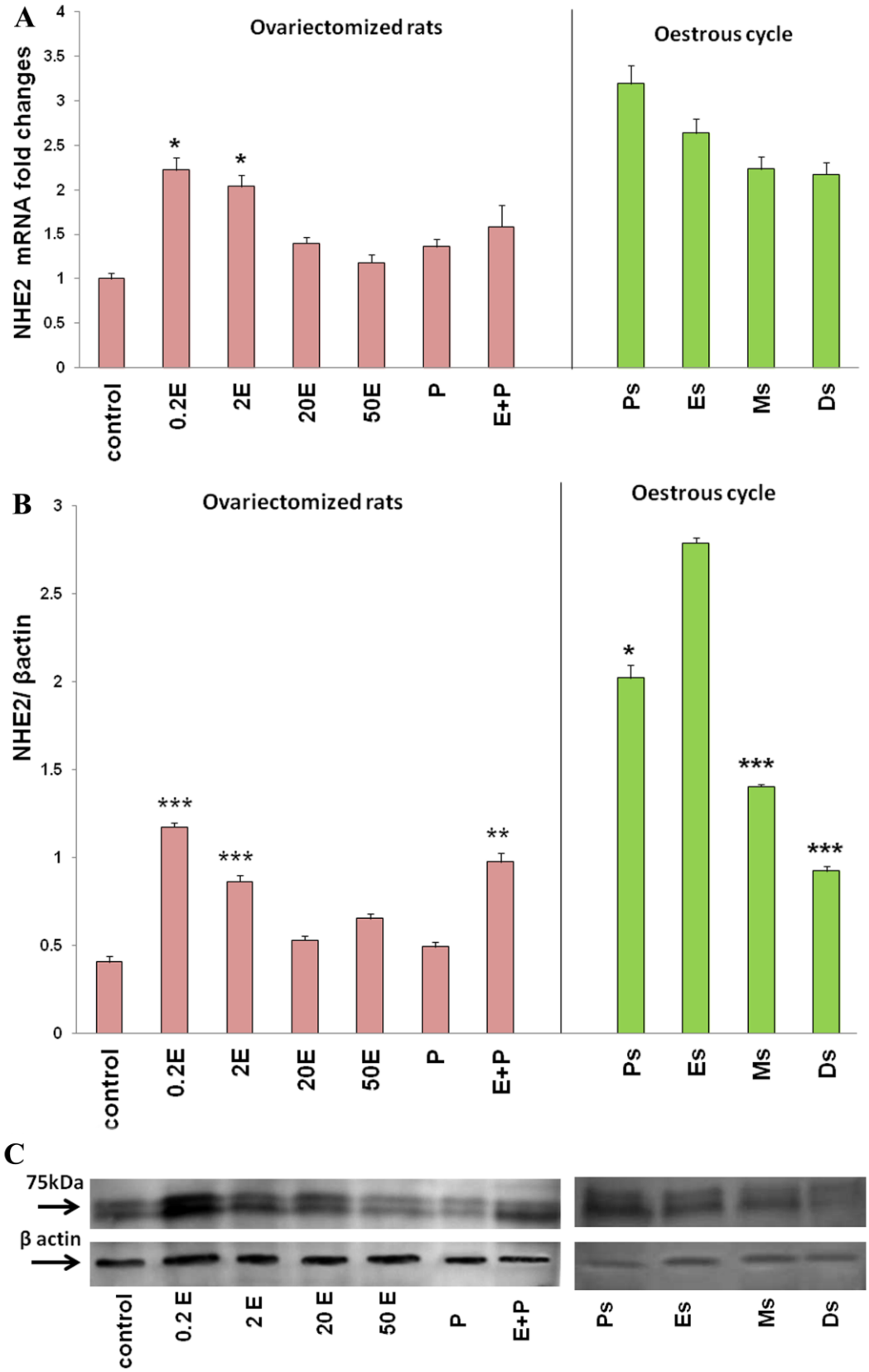



**Figure 5. 3** NHE2 mRNA (A) and protein (B) analysis and representative photos of western blot (C). 0.2E: 0.2µg estrogen, 2E: 2µg estrogen, 20 E: 20µg estrogen, 50E: 50 µg estrogen, P: 4mg progesterone, E+P: 0.2µg estrogen + 4mg progesterone. Ps: proestrus, Es: estrus, Ms: metestrus, Ds: diestrus. *, $P < 0.05$, **$P < 0.01$, ***$P < 0.001$.n=4 rats per group, data presented as mean±SEM. In ovariectomized rat comparison was done with control group however at oestrous cycle other groups were compared with Es.

### 5.2.3 Localization of NHE2

In figure 5.4 and Table 5.2, IHC results indicate that NHE2 protein was expressed mainly in the luminal and glandular epithelia under different steroid treatment although stromal and myometrial expression could also be seen at proestrus and estrus stages. Treatment with E resulted in a dose-dependent increase in the intensity of staining at both the apical and basolateral membrane of the luminal epithelia. The staining was the most intense under 20E and 50E. In the P treated group however, minimal apical staining could be seen, while moderate to intense staining could be observed in the E + P group. The distribution of NHE2 throughout the oestrous cycle correlates with the changing level of sex-steroid. The distribution was intense at proestrus and estrus, while minimal distribution could be seen at metestrus and diestrus.

**Table 5. 2** Semi quantification of NHE2 in the uterine luminal endometrium by IHC

| | Apical | Basal |
|---|---|---|
| control | -/+ | - |
| 0.2µg estrogen | ++ | + |
| 2µg estrogen | ++ | ++ |
| 20µg estrogen | +++ | ++ |
| 50µg estrogen | +++ | ++ |
| 4mg progesterone | + | - |
| 0.2µg estrogen +4mg progesterone | +++ | ++ |
| proestrus | +++ | +++ |
| estrus | +++ | +++ |
| metestrus | -/+ | - |
| diestrus | + | -/+ |

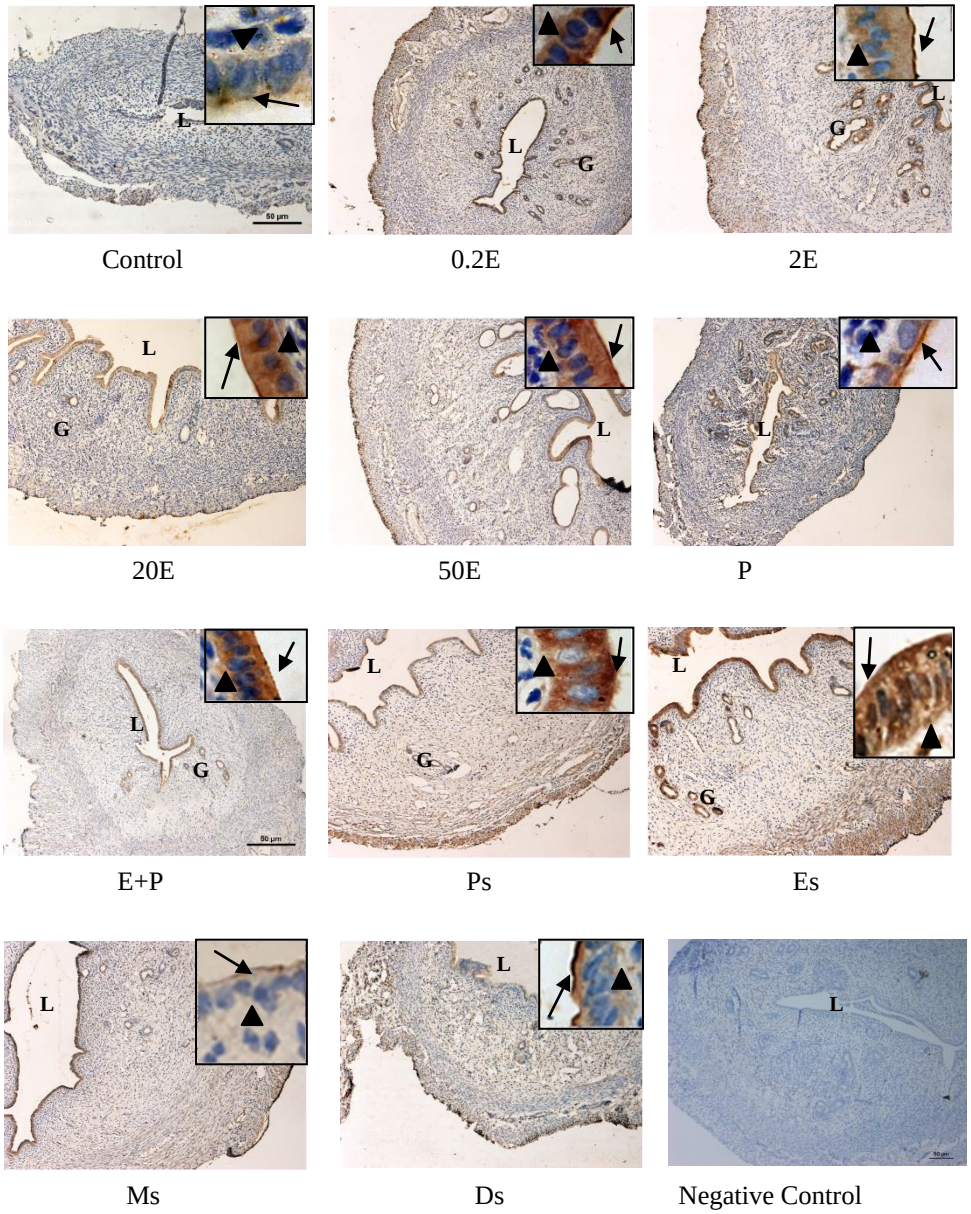



Figure 5.4 Immunodistribution of NHE2 in steroid treated ovariectomized rats and at different stages of oestrous cycle. Magnifications 10X and 100X (in the upper right corner). 0.2E: 0.2µg estrogen, 2µg estrogen, 20E: 20µg estrogen, 50E: 50µg oestrogen P: 4mg progesterone, E+P: 0.2µg estrogen +4mg progesterone, Ps: proestrus, Es: estrus, Ms: metestrus, Ds: diestrus. IHC with goat nonspecific immunoglobolin is negative control to check the specificity of antibodies. No staining was observed in this experiment. L: lumen, G: Gland, arrow shows luminal apical membrane, arrow head :basolateral membrane

## 5.3 NHE4 expression

### 5.3.1 NHE4 mRNA level and protein expression in ovariectomized rats

In figure 5.5.A, NHE4 mRNA level was the highest following treatment with E. Following 0.2E treatment, the expression level was increased by 14-fold, while treatment with 20E resulted in 20 fold increase in NHE4 mRNA level. P treatment led to significant inhibition in mRNA level in which there was only a 1.5 fold increase. Treatment with E followed by P also resulted in a significantly low NHE4 mRNA level (3.0 fold) as compared with 0.2E.

In figure 5.5.B, NHE4 protein was expressed the highest under E treatment. There was dose-dependent increase in the amount of NHE4 protein with increasing E doses (2.8 fold with 0.2E and 5.0 fold with 50E). P however causes a significant inhibition on NHE4 protein expression in which there was only a 0.9 fold increase. Treatment with E + P did not significantly differ from progesterone treated group.

### 5.3.2 NHE4 mRNA level and protein expression at oestrous cycle

Throughout the oestrous cycle, NHE4 mRNA level was the highest at proestrus (24 fold increase), followed by estrus (12 fold increase), which were consistent with a high endogenous E level. At metestrus and diestrus however, the level were significantly low which were 4.9 and 4.0 fold respectively. The expression of NHE4 mRNA at diestrus was 6 times lesser than at proestrus, suggesting that high endogenous P inhibits NHE4 mRNA expression (Figure 5.5.A).

At oestrous cycle phases however, NHE4 protein was expressed the highest at proestrus (6.0 fold) and estrus (4.65 fold). The lowest expression was at metestrus and diestrus (2.5 and 2.6 fold) respectively. The molecular weight for NHE4 is 70 kDa (Fig 5.5.C).

In general, under E influence, NHE4 mRNA is the most abundant isoforms. NHE4 mRNA level exceeds NHE2 mRNA by nearly 7-folds. Under P dominance however,

there was only a slight increase in NHE1 mRNA level as compared to E dominance state. NHE4 is the main protein isoform expressed under E effect although the expression of NHE2 isoform is also increased. Meanwhile, NHE1 isoform expression is slightly increased under P dominance.

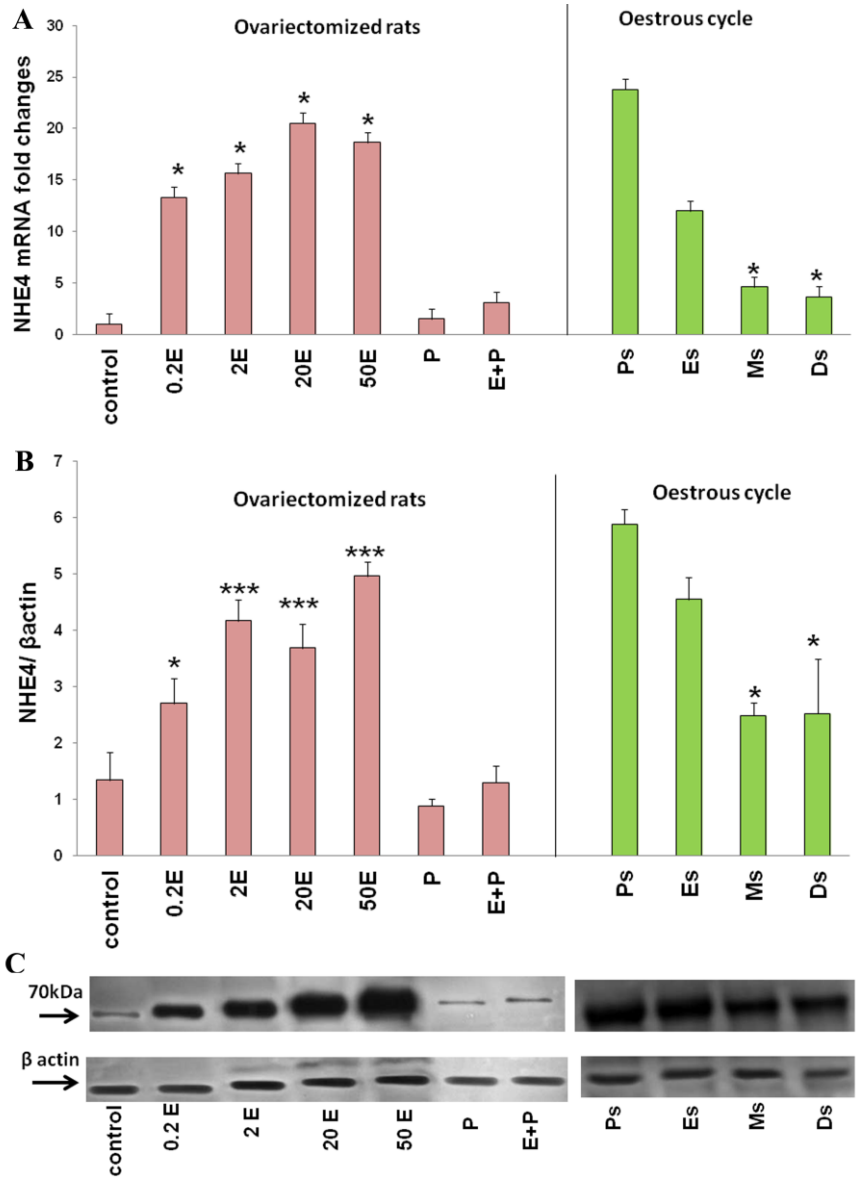



**Figure 5. 5** NHE4 mRNA and Protein analysis. Fluctuation in mRNA level (A), protein expression (B) and western blot photo (C) of NHE4. 0.2E: 0.2µg estrogen, 2E: 2µg estrogen, 20 E: 20µg estrogen, 50E: 50µg estrogen, P: 4mg progesterone, E+P: 0.2µg estrogen + 4 mg progesterone. Ps: proestrus, Es: estrus, Ms: metestrus, Ds: diestrus. *, $P < 0.05$, **$P < 0.01$, ***$P < 0.001$, indicate significant changes compared to control group in ovariectomized rats and compared to Es at different stages of the oestrous cycle. .n=4 rats per group, each has 3 technical replicate.

### 5.3.3 Localization of NHE4

In figure 5.6 and Table 5.3, NHE4 protein was seen to be distributed at both the luminal and glandular epithelia with intense staining observed under E treatment. Treatment with 50E however resulted in a moderate staining. P treatment resulted in a minimal staining while no staining was seen in the control group. Treatment with E + P however led to a mild to moderate staining. Meanwhile, throughout the oestrous cycle, the staining was very intense at proestrus and estrus, while a minimal staining was observed at diestrus.

**Table 5. 3** Semi quantification of NHE4 in the uterine luminal endometrium by IHC

| group | Apical | Basal |
|---|---|---|
| control | -/+ | - |
| 0.2µg estrogen | +++ | +++ |
| 2µg estrogen | +++ | ++ |
| 20µg estrogen | +++ | +++ |
| 50µg estrogen | + | -/+ |
| 4mg progesterone | + | -/+ |
| 0.2µg estrogen +4mg progesterone | ++ | ++ |
| proestrus | +++ | ++ |
| estrus | +++ | + |
| metestrus | +++ | -/+ |
| diestrus | + | -/+ |

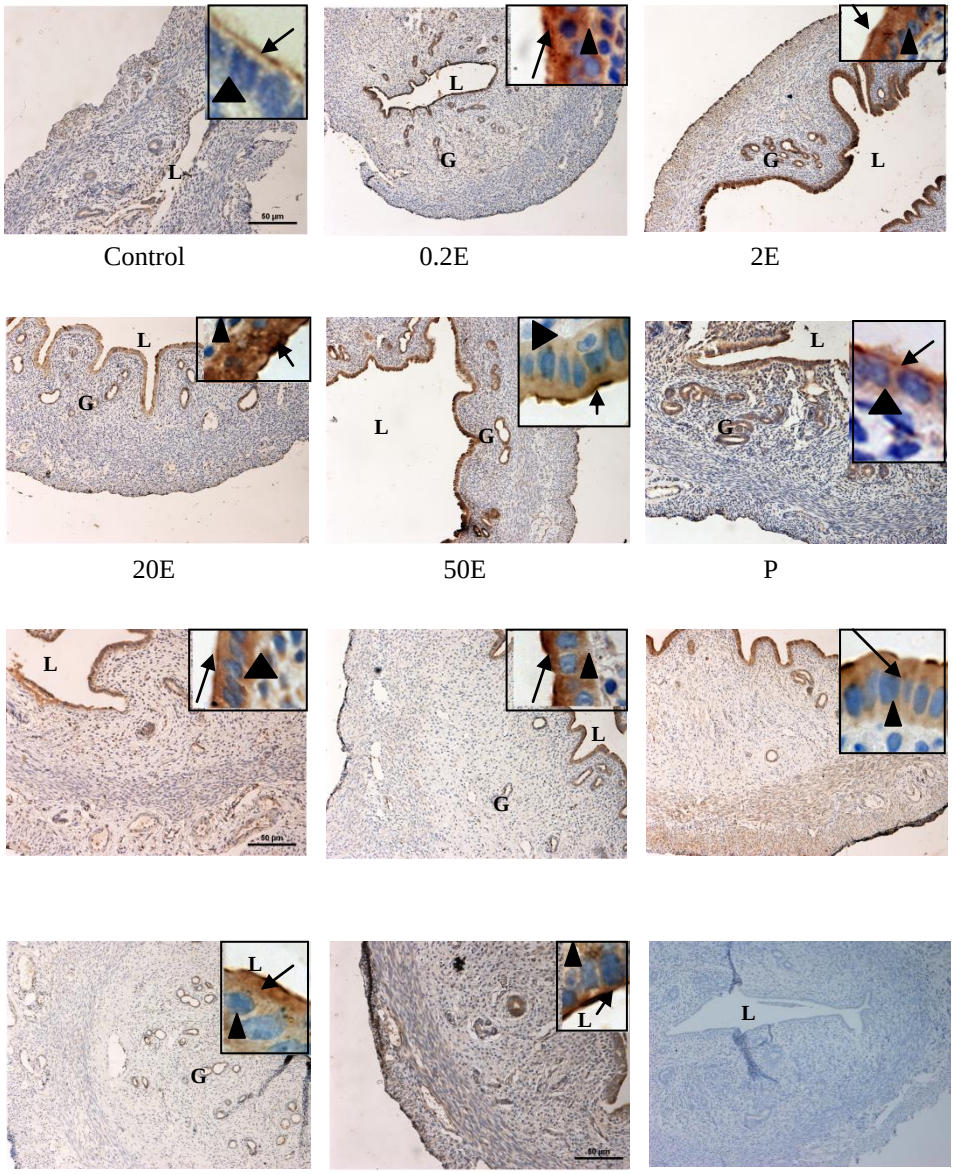



Figure 5.6 Immunodistribution of NHE4 in steroid treated ovariectomized rats and at different stages of oestrous cycle. Magnifications 10X and 100X (in the upper right corner). 0.2E: 0.2µg estrogen, 2µg estrogen, 20E: 20µg estrogen, 50E: 50µg estrogen P: 4mg progesterone, E+P: 0.2µg estrogen + 4mg progesterone, Ps: proestrus, Es: estrus, Ms: metestrus, Ds: diestrus. IHC with goat nonspecific immunoglobolin is negative control to check the specificity of antibodies. No staining was observed in this experiment. L: lumen, G: Gland, arrow shows luminal apical membrane, arrow head shows basolateral membrane

## 5.4 Discussion

Despite the reported existence of NHE1, 2 and 4 mRNAs in the endometrial epithelia from immature mice uteri (Wang, *et al.*, 2003b), the effect of sex-steroid and oestrous cycle phases on their expression remained largely unknown. Result showed that E up-regulates the expression of NHE4 and NHE2 mRNAs and proteins while P causes a concomitant increase in NHE1 mRNA and protein expression in the uterus, (ii) throughout the oestrous cycle, an increase in NHE1 mRNA and protein expression occur at diestrus while an increase in NHE2 and NHE4 mRNAs and proteins expression occur at proestrus and estrus, (iii) NHE4 is the most abundant NHE isoform expressed in the uterus under E stimulation and (iv) NHE2 and NHE4 are expressed at both the apical & basolateral membranes while NHE1 is predominantly expressed at the apical membrane of the luminal and glandular epithelia.

Differential expression of NHE isoforms under sex-steroid influence may explain the uterine fluid pH fluctuation (Figure 6.5.A) at different phases of the menstrual/oestrous cycle. We have shown that under E stimulation, an increase in the expression of basolaterally located NHE2 and NHE4 may mediate intracellular $H^+$ extrusion in exchange with $Na^+$. This is important in order to maintain the intracellular pH homeostasis associated with a continuous luminal $HCO_3^-$ secretion (Zhou, 2005). Intracellular $Na^+$ is generally believed to be pumped-out via the basolaterally located Na+/K+-ATPase (Turi, *et al.*, 1992).

In addition to an increase in the basolateral expression, we have also shown that E up-regulates the expression of NHE2 and NHE4 at the apical membrane of the luminal epithelia (Figure 5.4& 5.6). The role of apical NHE2 and NHE4 is unknown; however these isoforms may participate in luminal $Na^+$ secretion, which has been shown to be enhanced under E influence in mice (Wang, *et al.*, 2003b) and in rat (Figure 5.3 & 5.5) . It has long been established that E stimulates fluid and electrolytes secretion in the

endometrium (Clemetson et al., 1977). $Na^+$ secretion has been thought to occur exclusively via a leaky tight junction (Lee, *et al.*, 2000) since no transporters responsible for this process has been identified at the apical membrane of the endometrium. Aronson (1982) demonstrated that $Na^+$ efflux can be stimulated by intracellular $H^+$. Other findings in a native or NHE transfected cells suggested that NHE exchange activity is dependent on internal $H^+$ rather than external $Na^+$ or $H^+$ concentrations (Orlowski, 1993). Wakabayashi *et a*l (2003) reported that pHi dependent $Na^+$ efflux from cells expressing NHE1, NHE2 and NHE3 was stimulated by intracellular acidification and was completely inhibited by intracellular alkalinization. These observations support our hypothesis that under E dominance, apical NHE2 and NHE4 may catalyze a reverse mode of exchange of intracellular $Na^+$ for extracellular $H^+$, thus promoting $Na^+$ secretion into the uterine lumen. In addition to $HCO_3^-$ secretion via the CFTR and SLC26A6 (Figure 6.3.A& 6.6.A), which were known to be up-regulated by E (Figure 4.1& 4.3), $H^+$ reabsorption will further increased the pH of the uterine fluid. Meanwhile, E has also been reported to down-regulate the activity of basolaterally located $Na^+/K^+$-ATPase (Davis, *et al.*, 1978), which may further promotes the luminal $Na^+$ secretion.

We have also shown that NHE1 mRNA and protein expressions were up-regulated by P and at diestrus stage of the cycle (Figure 5.1). NHE1 was predominantly found at the apical membrane (Figure 5.2) supporting the view that this isoform participates in intraluminal fluid acidification under P influence (Figure 6.2.A). Additionally, the apically located NHE1 could also mediate $Na^+$ reabsorption in exchange with $H^+$. Typically, $Na^+$ reabsorption has been reported to occur via the ENaC (Salleh, *et al.*, 2005) , however in tissues lacking of ENaC such as epididymis, $Na^+$ reabsorption may also occur via the NHE (Leung, 2001). NHE mediated $Na^+$ reabsorption and $H^+$ secretion has been reported in the kidney, pancreas, intestine, colon, stomach, biliary

tree and middle ear (Arena, *et al.*, 2012; Choi, *et al.*, 2006; Hughes, *et al.*, 2010; Kirchhoff, *et al.*, 2003; Malakooti, 2011; Narins, *et al.*, 2004 ; Queiroz-Leite, 2011 ; Roussa, 2001). In human and mouse choroid plexuses, luminal NHE1 mediates net proton secretion into the cerebrospinal fluid (CSF) (Damkier, *et al.*, 2009). In addition to luminal fluid acidification, apical NHE has also been reported to participate in luminal fluid absorption driven by hypertonicity of the paracellular fluid secondary to $Na^+$ reabsorption. The role of NHE in luminal fluid loss has been described in the epididymis, kidney proximal tubules and intestinal epithelia (Noel & Pouyssegur, 1995; Wakabayashi, *et al.*, 1997). An increase in NHE1 expression at the apical membrane of the luminal epithelia could be responsible for the uterine luminal fluid acidification and secondary fluid loss observed under P influence (Figure 6.2& 6.5). Fluid loss may assist uterine closure, which brought the blastocyst in contact with the two opposing surfaces of the endometrium (Nilsson, 1972). The significance of P mediated increased in $H^+$ secretion is unknown; however it may help in creating an acidic environment required for blastocyst implantation (Nilsson, 1982). An increase in uterine fluid $H^+$ concentration may help to reduce the electrostatic repulsion force between the negatively charged endometrium and the blastocyst in addition to the blastocyst itself losing part of its negative charge at the time of implantation (Jerkinson, 1977). As mentioned earlier, the intracellular proton generation may occur via the action of CA. In order to maintain the intracellular pH homeostasis, $HCO_3^-$ is extruded into the plasma. The mechanisms underlying parallel $HCO_3^-$ extrusion via the basolateral membrane under P was not known, however it may involve the $HCO_3^-$ transporters such as SLC26A6 (Figure 4.4) and SLC26A4 (Suzuki, *et al.*, 2002). In addition, there is a possibility that P may also increase the expression and activity of endometrial $Na^+/K^+$-ATPase, which promotes luminal $Na^+$ reabsorption although there is no current evidence to support this. In conclusion, differential expression of NHE isoforms under

the effect of sex-steroid and throughout the oestrous cycle may have important implication towards the normal regulation of the uterine fluid pH and volume. Under E dominance, NHE2 and NHE4 up-regulation may assist uterine fluid alkalinization and intraluminal $Na^+$ secretion. Under P dominance however, NHE1 up-regulation may mediate intraluminal $H^+$ secretion in exchange with $Na^+$, thus facilitating secondary fluid loss which contributes to uterine closure that initiate the attachment phase of embryo implantation.

# Chapter 6

## Result & Discussion

# FUNCTIONAL STUDY

## 6.1 Changes in uterine fluid milieu in ovariectomized rats and rats throughout the oestrous cycle

### 6.1.1 Effect of exogenous sex steroid on the changes of uterine fluid

#### *6.1.1.1 Alteration of uterine fluid pH and volume*

Uterine luminal fluid pH in ovariectomized rats treated with peanut oil (control) was 7.54 and it increased with all doses of E in compared to the control group. There was no significance difference between 0.2E (pH 8.13) and 2E (pH 8.20), however administration of 20E (pH 8.28) and 50E (pH 8.38) increased pH significantly with respect to 0.2E that was considered as a physiologic dose. The pH in P (pH 7.38) treated group was less than that in control and was less than that in 0.2E. Another group treated with 3 days E followed by 3 days P (E+P), there was no significant difference (pH 7.58) with control or even with P treated rats, and pH was remarkably less than that in E groups (Figure 6.1.A). Thus, E increased the pH and provides a more alkaline environment while P reduced it with respect to control rats.

Comparison of uterine fluid secretion rate showed that in all E treated groups; the secretion rate was increased significantly. However, under the influence of P, secretion rate was less than that in the control group. The E+P group did not show any remarkable change with respect to the control group. 20 and 50E administration resulted in higher secretion rate compared to 0.2E. Furthermore secretion rate by 0.2E was noticeably more than P and E+P. Thus E led to higher secretion rate and increased uterine lumen volume, (Figure 6.1.B).

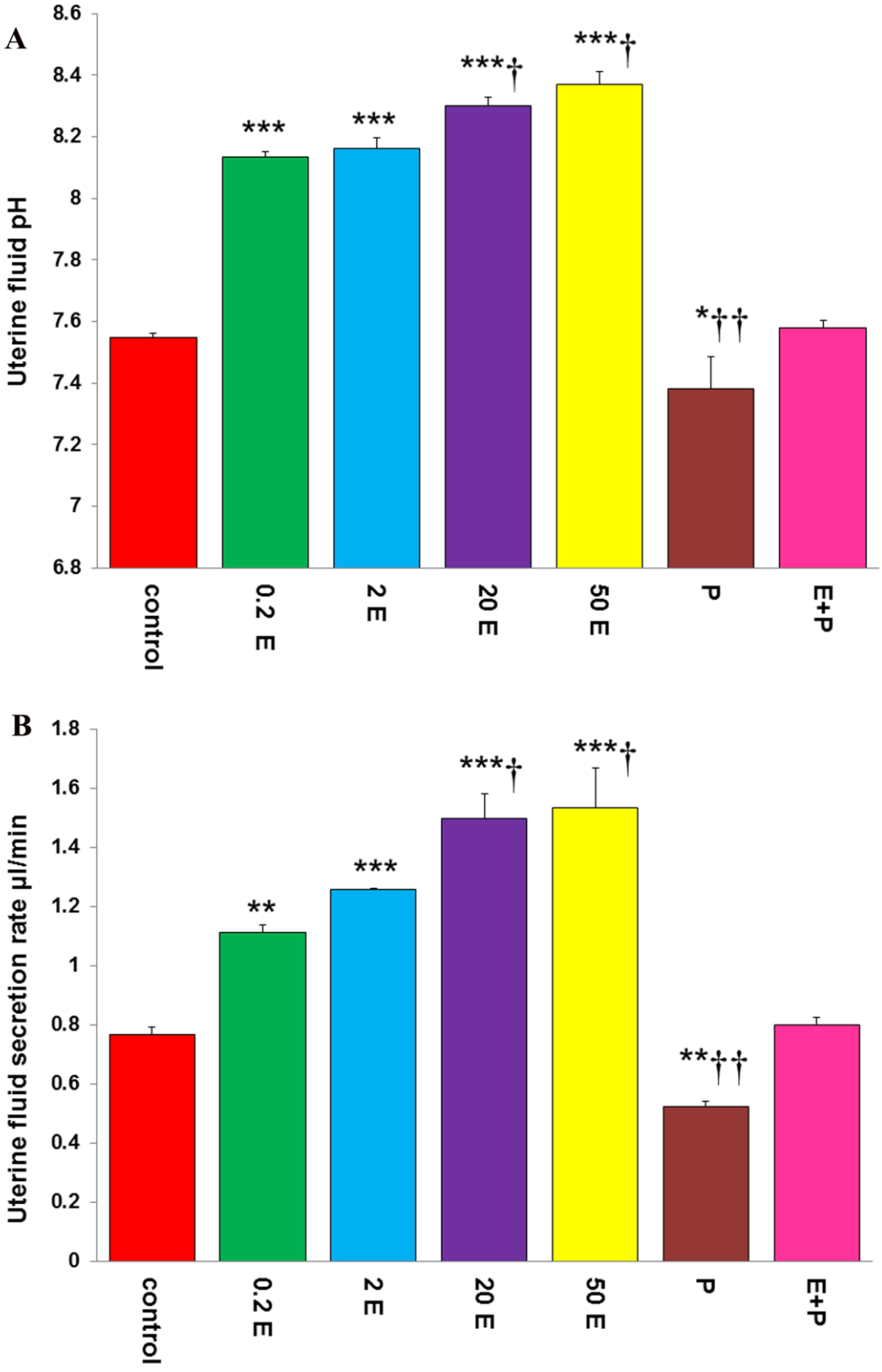



**Figure 6. 1** Effect of sex steroids on uterine fluid pH (A) and fluid secretion rate (B) in ovariectomized rats. 0.2E: 0.2µg estrogen, 2E: 2µg estrogen, 20E: 20µg estrogen, 50E: 50µg estrogen, P: 4mg progesterone, E+P: 0.2µg estrogen+ 4mg progesterone. *,† $p<0.05$, **,†† $p<0.01$ and *** $p<0.001$ with respect to control group. n=6 rats per group, data presented as mean ± SEM. * compared to control, † compared to 0.2E.

### *6.1.1.2 Effect of different inhibitors on the uterine fluid pH*

To evaluate the involvement of different proteins in uterine fluid changes under the influence of E, 0.2E was chosen as this dose consider as the physiologic dose (Salleh, *et al* 2005). Thus in following functional studies E treatment refers to 0.2E group.

The pH increases following E treatment was significantly inhibited by glibenclamide (CFTR inhibitor), DIDS (inhibitor of $HCO_3^-$ transporter) and ACTZ (CA inhibitor). EIPA (NHE inhibitor) however did not inhibit E-induced increase in uterine fluid pH. P treatment caused pH decrease which was antagonized by ACTZ and EIPA. In the E + P treated group however, the pH was significantly lower than the E-treated animals, however EIPA causes a significant increase in the pH ($p<0.01$), (Figure 6.2.A). Thus, $HCO_3^-$ transporter such as CFTR, $Cl/HCO_3^-$ exchanger and CA are involved in the changes of uterine fluid pH under the influence of E, while NHE and CA play a role in lower uterine fluid pH under the P influence.

### *6.1.1.3 Effect of different protein inhibitors on the uterine fluid secretion rate*

E treatment resulted in an increase in the rate of fluid secretion (Figure 6.2.B). This increase was inhibited by glibenclamide, DIDS and ACTZ while EIPA administration resulted in a significant increase in fluid secretion rate. On the other hand, P treatment resulted in a decrease in fluid secretion. The increase was significantly inhibited by EIPA and ACTZ. In the E+P group there was no difference in the rate of fluid secretion as compared to the control with only DIDS administration significantly reducing the rate of fluid secretion.

These results showed that under the influence of E, CFTR, CA and $Cl^-/HCO_3^-$ exchanger contributed in higher uterine fluid volume; in contrast NHE and CA are responsible for fluid changes under the influence of P.

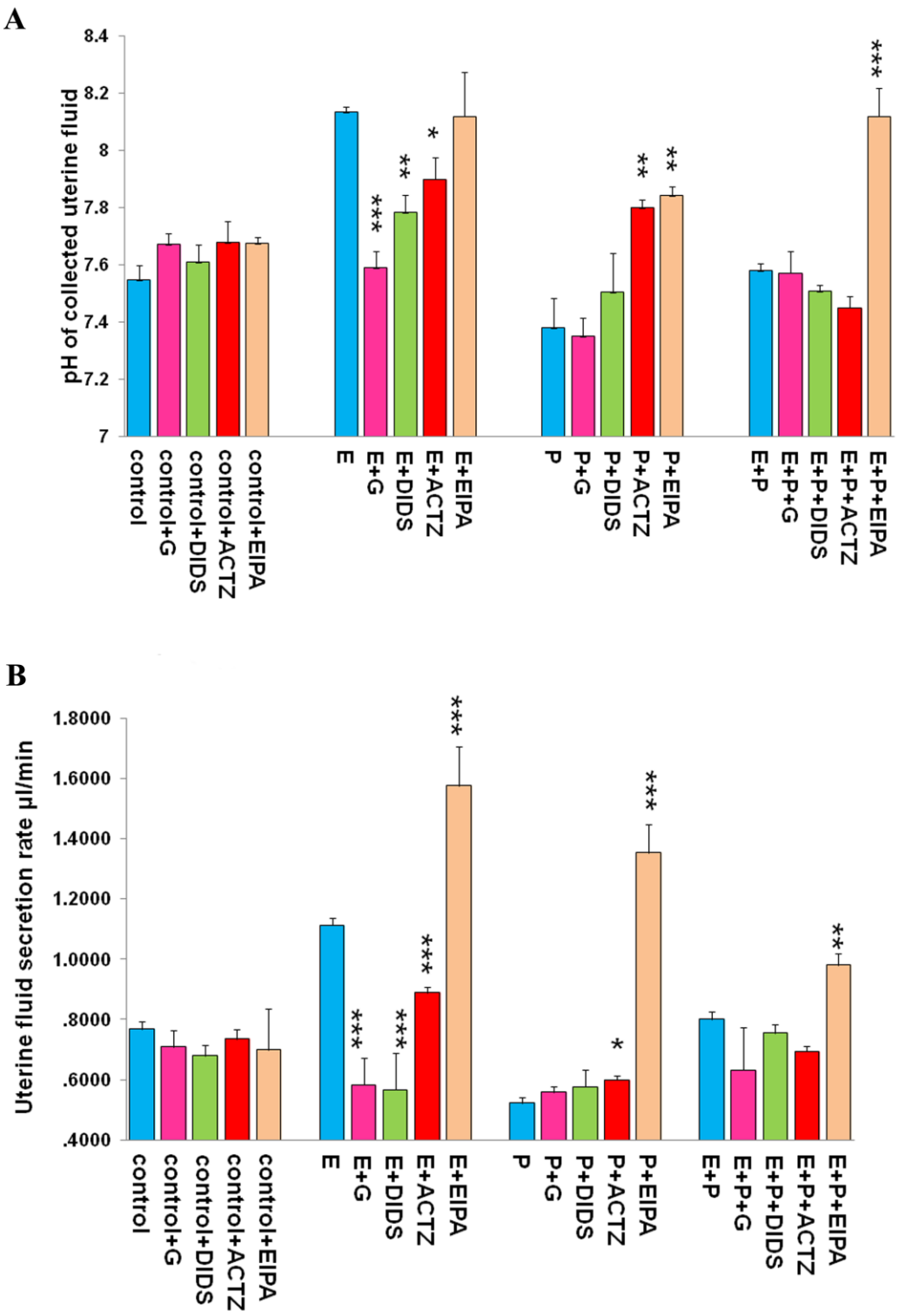



**Figure 6. 2** Effect of different inhibitors on the pH and fluid secretion rate of uterine fluid in ovariectomized rats. E: 0.2µg estrogen, P: 4mg progesterone, E+P: 0.2µg estrogen + 4mg progesterone. G: glibenclamide, ACTZ: acetazolamide, EIPA: 5-(N-Ethyl-N-isopropyl) amiloride, DIDS: 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic Acid, Disodium Salt. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. n=6 rats per group, data presented as mean±SEM.

### *6.1.1.4 Changes in $HCO_3^-$, $Cl^-$ concentration in ovariectomized rats*

There was an increase in $HCO_3^-$ secretion following E treatment (Figure 6.3.A), which was inhibited by glibenclamide, DIDS and ACTZ. P treatment however resulted in a significant reduction in uterine fluid $HCO_3^-$ concentration, which was antagonized by ACTZ and EIPA. No significant changes in $HCO_3^-$ concentration was seen in the E + P treated group as compared to the control or even following administration of inhibitors.

An increase in $Cl^-$ concentration following E treatment is inhibited by glibenclamide, DIDS and ACTZ (Figure 6.3.B). P treatment resulted in a lower $Cl^-$ content as compared to E. This was antagonized by ACTZ and EIPA. In the E + P group however, the $Cl^-$ concentration was significantly lesser than the E treated group. The addition of inhibitors however did not cause any significant effect on the $Cl^-$ concentration.

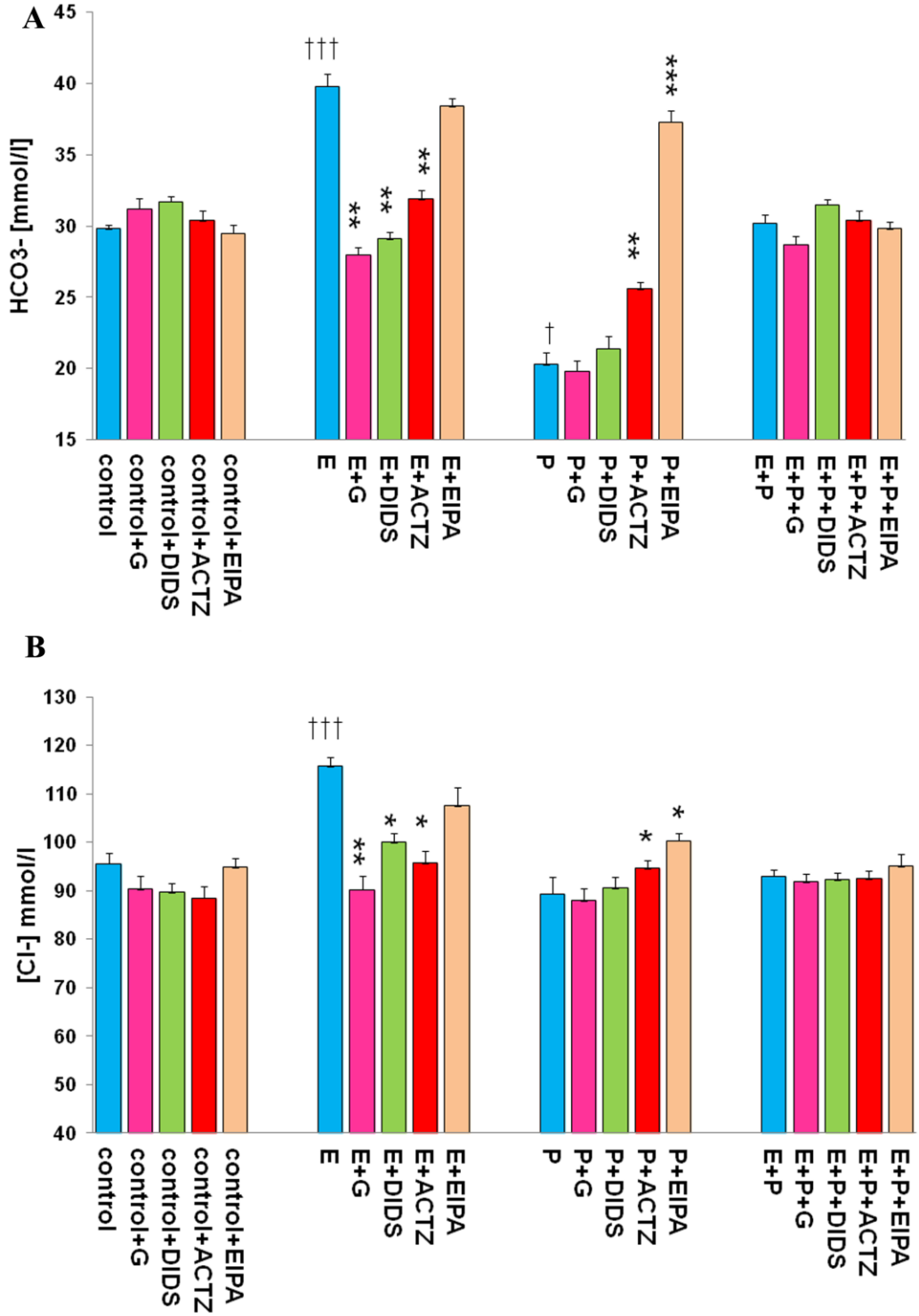



**Figure 6. 3** Changes in the $HCO_3^-$ (A) and $Cl^-$ (B) concentration, in the steroid replaced ovariectomized rats. E: 0.2µg estrogen, P: 4mg progesterone, E+P: 0.2µg estrogen + 4mg progesterone. G: glibenclamide, ACTZ: acetazolamide, EIPA: 5-(N-Ethyl-N-isopropyl) amiloride, DIDS: 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic Acid, Disodium Salt. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$, as compared with respected control group in each treatment. † compare between E, P and E+P with control group. n=6 rats per group, data presented as mean±SEM.

### *6.1.1.4 $Na^+$ concentration in the steroid treated ovariectomized rats*

$Na^+$ concentration was increased under E treatment. This enhancement was inhibited by glibenclamide and DIDS. P treatment resulted in a decrease in $Na^+$ concentration, which was inhibited by EIPA and ACTZ. In the E + P group, EIPA antagonized the reduction in $Na^+$ concentration.

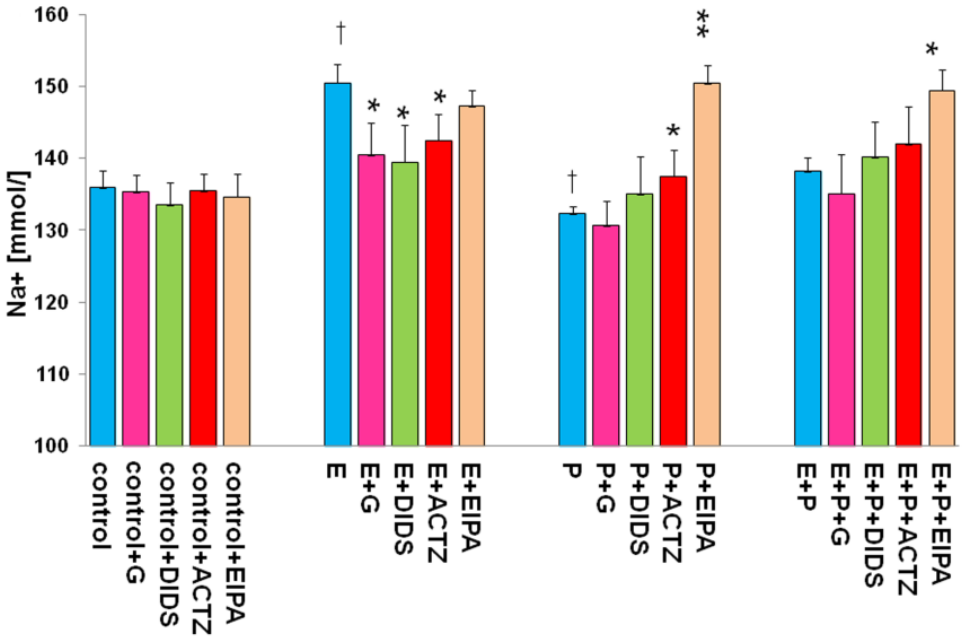



**Figure 6. 4** Changes in the $Na^+$ level of the uterine fluid in ovariectomized rats. E: 0.2µg estrogen, P: 4mg progesterone, E+P: 0.2µg estrogen + 4mg progesterone. G: glibenclamide, ACTZ: acetazolamide, EIPA: 5-(N-Ethyl-N-isopropyl) amiloride, DIDS: 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic Acid, Disodium Salt. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$, as compared with respected control group in each treatment. † compare between E, P and E+P with control group. n=6 rats per group, data presented as mean±SEM.

### 6.1.2 Uterine fluid environment throughout the oestrous cycle

#### *6.1.2.1 Fluctuation in the pH of uterine fluid throughout the oestrous cycle*

Throughout the phases of oestrous cycle, high pH was observed at proestrus (7.93) and estrus (7.95) consistent with a high circulating E concentration, while low pH was seen at diestrus (7.67) and metestrus (7.75) consistent with a high circulating P concentration. The high uterine fluid pH at proestrus and estrus phases was inhibited by glibenclamide, DIDS and ACTZ (Figure 6.5.A). At diestrus however, a reduction in the pH was significantly antagonized by ACTZ and EIPA. The pH reduction seen at metestrus was significantly antagonized by EIPA ($p<0.01$), (Figure 6.5.A).

#### *6.2.2 Effect of endogenous steroid on secretion rate of uterine fluid*

There was an increase in the rate of fluid secretion at proestrus and estrus stages of the cycle. The reduction in the rate of fluid secretion was inhibited by glibenclamide, DIDS and ACTZ; while EIPA causes an increase in fluid secretion at both proestrus and estrus. A lower fluid secretion rate was observed at diestrus and metestrus and this was antagonized by ACTZ as well as EIPA and EIPA alone respectively (Figure 6.5.B).

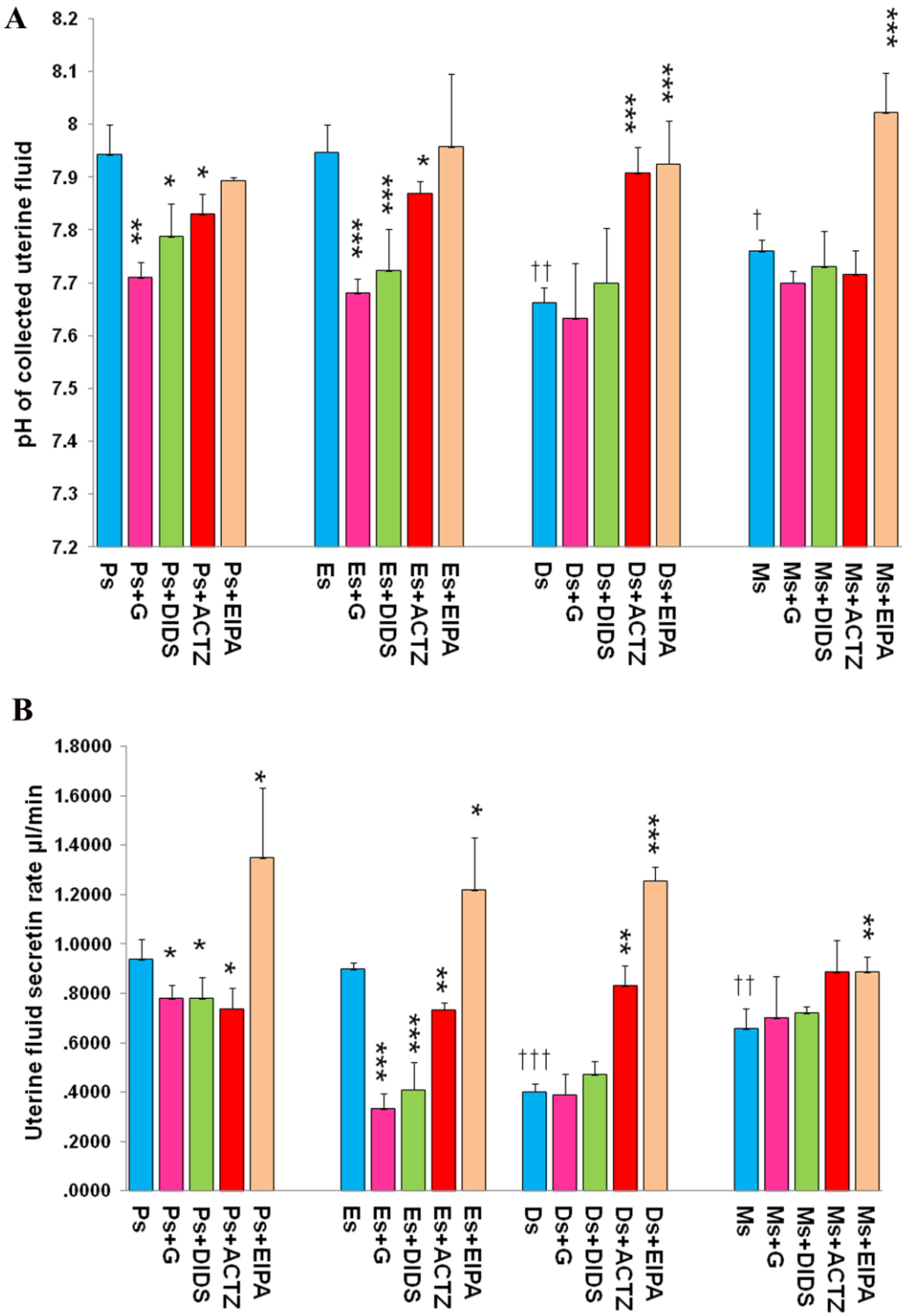



**Figure 6. 5** Changes in the pH (A) and fluid secretion rate, (B) at different stages of the oestrous cycle and effect of different protein inhibitors. Ps: proestrus, Es: estrus, Ms: metestrus, Ds: diestrus. G:glibenclamide, ACTZ: acetazolamide, EIPA: 5-(N-Ethyl-N-isopropyl) amiloride, DIDS: 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic Acid, Disodium Salt.. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. * shows the comparison with respected control in each stages of the oestrous cycle, † shows the comparison among Ps, Ms and Ds with Es. n=6 rats per group, data presented as mean± SEM.

### *6.1.2.3 Alteration in the $HCO_3^-$ and $Cl^-$ content of the uterine fluid during oestrous cycle*

There was an increase in $HCO_3^-$ concentration at proestrus and estrus stages, and this increase was significantly inhibited by glibenclamide (CFTR inhibitor), DIDS and ACTZ. $HCO_3^-$ concentration was lowest at diestrus, and this was antagonized by ACTZ and EIPA. At metestrus however, the decrease in $HCO_3^-$ concentration was inhibited by EIPA, (Figure 6.6A).

Chloride concentration was highest at proestrus and estrus, and lowest at diestrus. At metestrus however, $Cl^-$ concentration was significantly lower than at proestrus. Addition of glibenclamide resulted in a significant decrease in $Cl^-$ content at proestrus and estrus, while DIDS administration resulted in an increase in $Cl^-$ concentration. ACTZ and EIPA meanwhile caused an increase in $Cl^-$ content at diestrus, but not at metestrus.

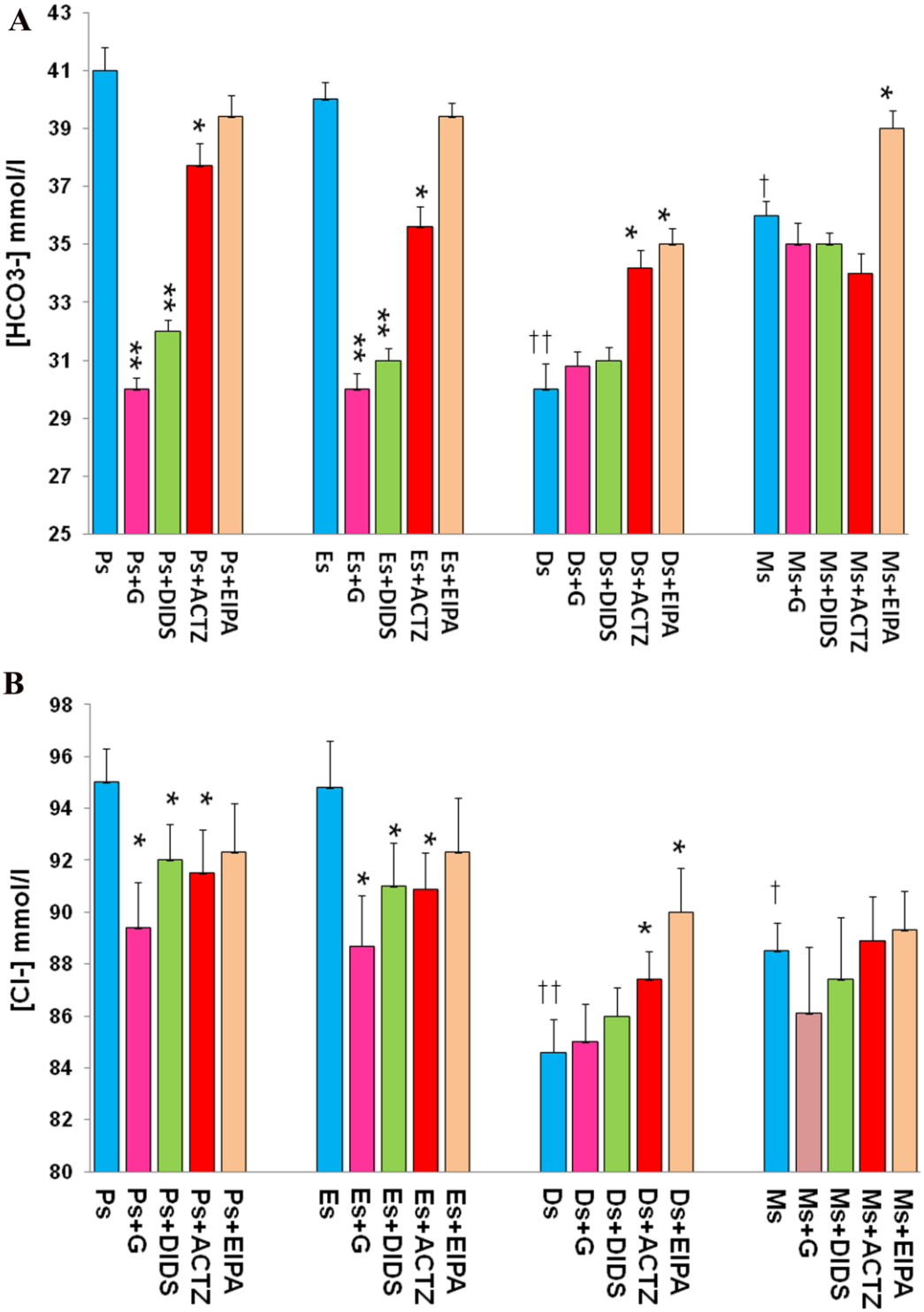



**Figure 6. 6** $HCO_3^-$ (A) and $Cl^-$ (B) changes throughout the oestrous cycle Ps: proestrus, Es: estrus, Ms: metestrus, Ds: diestrus. G:glibenclamide, ACTZ: acetazolamide, EIPA: 5-(N-Ethyl-N-isopropyl) amiloride, DIDS: 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic Acid, Disodium Salt. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$, * shows the comparison with respected control in each stages of the oestrous cycle, † shows the comparison among Ps, Ms and Ds with Es. n=6 per group, data presented as mean ±SEM.

### *6.1.2.4 Effect of different inhibitors in $Na^+$ content of the uterine fluid at oestrous cycle*

In figure 6.7, $Na^+$ concentration was the highest at proestrus and estrus which was inhibited by glibenclamide and DIDS. $Na^+$ concentration was relatively lower at diestrus and metestrus, which is antagonized by EIPA and ACTZ and EIPA alone respectively.

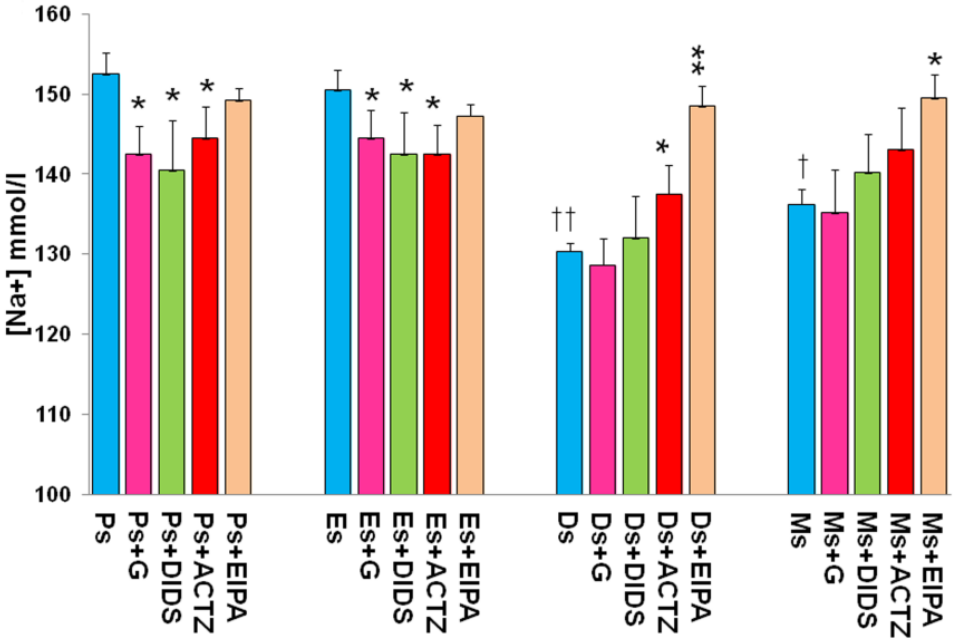



**Figure 6. 7** $Na^+$ concentration in the uterine fluid during different stages of the oestrous cycle. Ps: proestrus, Es: estrus, Ms: metestrus, Ds: diestrus. G:glibenclamide, ACTZ: acetazolamide, EIPA: 5-(N-Ethyl-N-isopropyl) amiloride, DIDS: 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic Acid, Disodium Salt. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$, * shows the comparison with respected control in each stages of the oestrous cycle, † shows the comparison among Ps, Ms and Ds with Es. n=6 per group, data presented as mean±SEM

## 6.2 Discussion

Many methods have been employed for collection of uterine fluid, including cannulation in which a cannula is installed by surgery in the uterine tube. This method is suitable for big animals such as cow, ewe, rabbit and monkey. The uterine fluid can be collected over the time of few days up to a few months (Aguilar & Reyle, 2005; Grippo, *et al.,* 1995). The most commonly method used in rat and mouse is uterine flushing, however in this method fluid probably get contaminated by plasma and interstitial fluid (Milligan &martin, 1984). Another method to observe the changes of uterine fluid is cultured endometrial cell (He, *et al.*, 2010; Wang, *et al.*, 2002) whereby protein expression or function maybe lost during cell culture (Downing, *et al*., 1997). Thus in this study the uterus was perfused and the fluid was collected at the cervical end of uterus as has been described before (Salleh, *et al.*, 2005), whereby the uterine fluid has less chance of manipulation during sample collection.

This study reveals the participation of multiple proteins in uterine fluid pH regulation under different effect of sex-steroid and at different phases of oestrous cycle in *in-vivo* model. We have shown that uterine fluid pH control is linked to the regulation of uterine fluid volume and electrolytes. Changes in uterine fluid pH and volume were observed under higher dose of E (Figure 6.1). Although uterine fluid pH was found to be alkaline at around the time of ovulation (Murdoch & White, 1971). There is currently lack of information on the pH status in the post-ovulatory period as well as at early phase of the cycle. We have therefore provided the first direct evidence on uterine fluid pH fluctuation throughout the oestrous cycle, being the highest at proestrus and estrus and the lowest at diestrus (Figure 6.5A), consistent with an increasing E and P level respectively. This pH changes is parallel with the observed changes in $HCO_3^-$ concentration under different effect of sex-steroid and at different phases of the cycle (Figure 6.5.B). The highest $HCO_3^-$ concentration observed under E, was approximately

40 mmol/l (Figure 6.3.A & 6.6.A) is consistent with the reported $HCO_3^-$ concentration at the follicular phase (35 mmol/l) in rhesus monkey (Macaca mulatta) which peaks-up to more than 90 mM at around the time of ovulation (Maas, *et al.*, 1977). E dependent $HCO_3^-$ secretion has also been reported in mice and human duodenum (Smith, *et al.*, 2008; Tuo, *et al.*, 2011). In addition to the changes in $HCO_3^-$, uterine fluid volume, $Cl^-$ and $Na^+$ content (Figure 6.3&4 and 6.6&7) have also been found to be reduced following P treatment and at diestrus as oppose to an increase in their level under the effect of E and at estrus and proestrus phases.

This study indicates that CFTR is involved in uterine fluid alkalinization under the influence of E (Figure 6.2.A& 6.3.A). In addition to being a $Cl^-$ channel, CFTR has been shown to transport $HCO_3^-$ (Hug, *et al.*, 2003). Indeed a parallel reduction in the uterine fluid pH, $HCO_3^-$ and $Cl^-$ concentrations was observed following glibenclamide administration (Figure 6.2-4). This is confirmed the notion that CFTR participates in uterine fluid secretion (Chan, *et al.*, 2009), as a reduction in uterine fluid volume occurred following glibenclamide administration (Figure 6.2.B). CFTR activation resulted in luminal $Cl^-$ efflux which then drives fluid secretion (6.3.B). CFTR mediated fluid secretion has been reported in the airway (Shan, *et al.*, 2012) and kidney epithelial cells (Wallace, *et al.*, 1996). Apart from stimulating fluid secretion via CFTR, E has also been reported to induce the formation of a leaky tight junction, through which paracellular fluid movement occurs, thus contributing to the intrauterine fluid accumulation (Lindsay & Murphy, 2006).

As mentioned above, glibenclamide inhibition on $HCO_3^-$ secretion indicates CFTR involvement in uterine $HCO_3^-$ transport. This may resemble the mechanism in the pancreas, where CFTR has been reported to function together with the $Cl^-/HCO_3^-$ exchangers (SLC26A6) in mediating $HCO_3^-$ secretion, which contributes to pancreatic fluid alkalinization (Hug, *et al.*, 2003; Wang, *et al.*, 2006). As a major $Cl^-$ channel,

CFTR has been reported to mediate $Cl^-$ secretion under E stimulation (Salleh, *et al.*, 2005). A high uterine fluid $Cl^-$ concentration has been reported at around the time of implantation in rats (Nilsson & Ljung, 1985). CFTR may be involved indirectly in luminal $Na^+$ secretion under E, which is electrogenically driven by a high intraluminal $Cl^-$ concentration (Crowell, 2009). Immunohistochemistry confirmed CFTR distribution in the luminal and glandular epithelia following E treatment and at estrus (Figure 4.2), consistent with the previous findings (Chan, *et al.*, 2009; Chan, *et al.*, 2002; Salleh, *et al.*, 2005). Yang, *et al* (2004) meanwhile reported a stromal CFTR expression prior to implantation in rats. While stromal CFTR could be involved in decidualization, epithelial CFTR plays an important role in uterine fluid volume, pH and $Cl^-$ regulation (Figure 6.2& 6.3). Following P treatment and at diestrus, CFTR expression was found to be reduced (Figure 4.1), which explains a decrease in the volume, $Cl^-$ and $HCO_3^-$ concentrations of the uterine fluid. Additionally, lack of glibenclamide inhibition confirmed this.

The observed effects of DIDS suggest the involvement of either $Cl^-/HCO_3^-$ exchanger (SLC26A6) or NBC (SLC24A4) in the pH increase following E treatment, at estrus and proestrus (Figure 6.1.A) as DIDS is a non-specific inhibitor of these proteins (Chen, *et al.*, 2008; He, *et al.*, 2010; Shahidullah, *et al.*, 2009). A reduction in the pH following DIDS administration was lesser than that observed following glibenclamide administration (Figure 6.2.A), suggesting that CFTR is the main protein involved in uterine fluid alkalinization. In view of this, SLC26A6 and/or SLC4A4 may complement CFTR function in the uterus. Following DIDS administration, uterine $HCO_3^-$ concentration was found to be reduced (Figure 6.3.A & 6.6.A) under the influence of E and during estrus, which indicates $HCO_3^-$ secretion occur via these anion exchangers (SLC4A4 and/or SLC26A6) under these conditions. Meanwhile, SLC26A6 has been reported to mediate uterine $HCO_3^-$ secretion in an *in vitro* study in endometrial cells

obtained from mice treated with E and at estrus (He, *et al.*, 2010). Our findings which indicate that SLC26A6 was present in the luminal and glandular epithelia under E dominance (Figure 4.4) confirmed the involvement of this protein in uterine $HCO_3^-$ secretion. Meanwhile, SLC26A6 has also been reported to be involved in mediating $HCO_3^-$ secretion in other tissue such as pancreatic duct (Ishiguro, *et al.*, 2007). Other than SLC26A6, the involvement of apical SLC4A4 cannot be excluded (Figure 4.6).

An inhibition of the volume (Figure 6.2.B& 6.5.B), $Cl^-$ (Figure 6.3.B& 6.6.B) and $Na^+$ (Figure 6.4& 6.7) increase following DIDS administration under E and at estrus suggest the involvement of SLC26A6 or SLC4A4 in mediating this effect. The involvement of the former is more likely than the latter since its uterine expression of SLC26A6 has been reported to be influenced by sex-steroid. Although SLC26A6 does not directly involved in fluid and $Cl^-$ secretion, these effects may occur via CFTR inhibition. CFTR activity can be affected by SLC26A6 (Ko, *et al.*, 2004). Interactions between these two proteins are likely as they have been found to be co-expressed in the uterus under E and at estrus (Figure 4.1& 4.3). Interactions between CFTR and SLC26A6 have been reported in the pancreas (Lohi, *et al.*, 2003), salivary glands, airways, vas deferens and intestine (Wang, *et al.*, 2006). SLC26A6 inhibition will result in a decreased CFTR activity (Ko, *et al.*, 2002) which may explain a reduction in volume (Figure 6.2.B& 6.5.B), $Cl^-$ (Figure 6.3.B& 6.6.B) and $Na^+$ (Figure 6.4& 6.7) concentrations following E treatment and at estrus and proestrus. Meanwhile, lack of DIDS inhibition on the volume, pH, $HCO_3^-$, $Na^+$ and $Cl^-$ decrease following P treatment and at diestrus (Figure 6.2-6.7) confirmed that the expression and activity of this protein was reduced under these conditions (Figure 6.3).

Administration of EIPA in the E-treated group and at estrus did not have any effect of the uterine fluid pH (Figure 6.2.A& 6.5.A), however it is resulted in a significant increase in volume (Figure 6.2.B& 6.5.B) which suggests that other transporter apart

from NHE is involved in mediating this effect. EIPA has been shown to inhibit the ENaC (Masereel, *et al.*, 2003), that explain the excessive increase in uterine fluid volume under these conditions. ENaC is a negative regulator of CFTR (Stutts, *et al.*, 1995). ENaC inhibition will augment CFTR activity, resulting in an increase in fluid secretion. EIPA did not affect $HCO_3^-$ concentration (Figure6.3.A& 6.6.A) although a reduction in $Na^+$ (Figure 6.4&6.7) and $Cl^-$ (Figure 6.3.B&6.6.B) concentrations could be seen, possibly due to increase in volume of uterine fluid (Figure 6.2.B&6.5.B). Lack of EIPA effect on $HCO_3^-$ secretion under E could be due to the basolateral NHE localization in mouse (Wang, *et al.*, 2003b) and rat (Figure 5.4&5.6), which participates in the exchange of external $Na^+$ with internal $H^+$ (Dunham, *et al.*, 2004; Orlowski & Grinstein, 2004).

Meanwhile, EIPA-related increase in uterine fluid pH following P treatment (Figure 6.2.A) and at diestrus (Figure 6.5.A) indicates the involvement of NHE in uterine fluid acidification. This was confirmed by IHC in which NHE-1 expression was increased under the influence of P and at diestrus, mainly at the apical membrane (Figure 5.2), which mediates $H^+$ secretion. In addition, CFTR (Figure 4.1&4.2) and SLC26A6 (Figure 4.3&4.4) expressions were also found to be markedly reduced under these conditions, resulting in decrease in $HCO_3^-$ secretion, which will further contribute to a reduction in the uterine fluid pH (Figure 6.1A&6.5.A). EIPA has also been shown to inhibit a reduction in the volume, $HCO_3^-$, $Cl^-$ and $Na^+$ concentrations under the effect of P (Figure 6.2.B, 6.3& 6.4) and at diestrus (Figure 6.5.B, 6.6&6.7). EIPA-related increase in fluid volume suggesting that P-induced fluid loss may occur via ENaC and/or NHE. Progesterone-induced fluid absorption secondary to $Na^+$ reabsorption has been documented in the uterus (Naftalin, *et al.*, 2002), which is mediated via ENaC (Chan, *et al.*, 2002; Salleh, *et al.*, 2005). Although ENaC serves as a major pathway for uterine fluid reabsorption under the P effect (Salleh, *et al.*, 2005) NHE could provide an

alternative pathway for fluid loss as has been reported in the epididymis (Leung, 2001). $Na^+$ reabsorption via ENaC or/and NHE will drive electrogenic $Cl^-$ influx, creating an osmotic gradient necessary for $H_2O$ absorption (Naftalin, *et al.*, 1999). This may explain the observed reduction in $Na^+$ and $Cl^-$ concentrations under P (Figure 6.3.B&6.4) and at diestrus (Figure6.6.B&6.7). Meanwhile, P has also been reported to up-regulate the expression of aquaporin (AQP) channel in the endometrium (Lindsay & Murphy, 2007) as well as inducing the formation of a "tight" tight junction which would further prevents paracellular fluid movements (Li, *et al.*, 1994; Lindsay & Murphy, 2006).

Results also showed that ACTZ administration antagonized the pH changes in both E and P treated groups (Figure 6.2.A). ACTZ inhibits both intracellular CAII and membrane-bound CAXII isoforms (Berg 2004). Intracellular CA II catalyzes the conversion of $CO_2$ and $H_2O$ to $H_2CO_3$, which result in the generation of $H^+$ and $HCO_3^-$ (Boron, 2010). Meanwhile, membrane bound CA XII catalyzes the conversion of extracellular $H^+$ and $HCO_3^-$ to $CO_2$ and $H_2O$ (Karhumaa, *et al.*, 2001a). The effect of ACTZ on uterine fluid pH following E treatment, at estrus and proestrus suggest the involvement of CA in uterine fluid alkalinization (Figure 6.2.A& 6.5.A). A slight decrease in the pH following ACTZ as compared to DIDS and glibenclamide administration suggest that CA play a less important role in pH increase as compared to SLC26A6 and CFTR under the same conditions. These findings also conclude that the plasma-derived $HCO_3^-$ is a major contributor to uterine fluid $HCO_3^-$ rather than the intracellular $HCO_3^-$ generation involving CA.

Intracellular CA II catalyzes the reaction that results in $HCO_3^-$ synthesis (Karhumaa, *et al.*, 2001a), which will then be secreted via CFTR and SLC26A6 (Figure 4.2&4.4). Meanwhile, concomitant $H^+$ production will be secreted via the basolateral NHE (Chan, *et al.*, 2009; Wang, *et al.*, 2003b) in order to maintain the intracellular pH homeostasis. In addition to participating in the pH control, CA may also be involved in uterine fluid

secretion. The involvement of CA in anion and fluid secretion has been reported in the cerebrospinal fluid (Maren & Broder, 1970). This increase in uterine fluid secretion may occur indirectly via CFTR. The inhibitory effects of ACTZ on E-stimulated increase in $Na^+$ and $Cl^-$ concentrations also indicates indirect involvement of CA in electrolytes regulations (Figure 6.3.B&6.4). In this study, CA II isoform is present mainly in the glandular epithelia under the effect of E and at estrus (Figure 4.8).

In addition to participating in uterine fluid alkalinization under the influence of E, CA also participates in the uterine fluid acidification under P effect and at diestrus (Figure 6.2.A&6.5.A). This may involve the intracellular CA II and a membrane bound CA XII. The latter isoenzyme participates in intraluminal $HCO_3^-$ buffering, resulting in $H_2CO_3$ generation, which ultimately formed $CO_2$ and $H_2O$ (Ivanov, *et al.*, 2001). $CO_2$ will then diffuse into the cell and is converted into $H_2CO_3$ by intracellular CAII, which ultimately dissociates into $H^+$ and $HCO_3^-$ (Boron, 2010). $H^+$ will then be secreted into the lumen via NHE-1, which expression is increased under the influence of P and at diestrus (Figure 4.7). Meanwhile, basolateral $HCO_3^-$ secretion may occur via the basolaterally located anion exchanger such as NBC (SLC4A4) and SLC26A6 to maintain the intracellular pH homeostasis (Table 4.2&4.3). These processes are required to maintain a continuous $H^+$ secretion which results in uterine fluid acidification as observed under P influence.

An increase in the volume (Figure 6.2.B), $Na^+$ (Figure 6.4), $Cl^-$ and $HCO_3^-$ (Figure 6.3) concentrations following ACTZ administration under P suggested that CA is involved in mediating these changes. A decreased in $HCO_3^-$ concentration following P treatment and at diestrus is a direct consequence of an increased in $H^+$ secretion. The mechanism underlying an increase in uterine fluid volume following ACTZ administration is however unknown, although there is a possibility that ACTZ may inhibit the AQP channels (Berg, *et al.*, 2004) may inhibit fluid reabsorption induced by P, thus resulting

in a volume increase. The observed increase in $Na^+$ concentration following ACTZ administration indicates that CA may indirectly involve in $Na^+$ reabsorption via the NHE, which will then induced electrogenic $Cl^-$ absorption, thus explaining the increased in luminal $Cl^-$ concentration following ACTZ administration (Figure 6.3.B&6.6.B). The presence of CA isoforms following P treatment and at diestrus was confirmed by immunostaining, in which CA II was found mainly in the glandular epithelia (Figure 4.8) while CA XII was present mainly in the luminal as well as glandular epithelia (Figure 4.10).

Treatment with E followed by P did not result in any significant changes in the uterine fluid parameters as compared to the control. The results obtained following administration of various proteins inhibitors could not conclude the involvement of CFTR, SLC4A4/SLC26A6 and CA apart from a limited observation which indicate that NHE or ENaC may participate in regulating the uterine fluid pH and $Na^+$ concentration. In view of this, a more complex mechanism may be involved under this condition. Meanwhile, the changes seen at metestrus reflect the transitions between estrus to diestrus phases. Our findings which indicate that EIPA inhibits the changes at metestrus suggest the participation of NHE and/or ENaC in these processes. In conclusion, the present study provides an insight into the possible mechanisms underlying the concomitant changes in the pH, volume, and electrolytes content of the uterine luminal fluid. By using an *in-vivo* model, we have successfully provided evidence on the relationship between the uterine fluid pH, volume and electrolytes concentration changes under different sex-steroid influence. This understanding is important for normal reproduction as any deviation from these parameters could have a significant impact on fertility.

In this study expression of protein channel that involved in uterine fluid changes has been investigated under high estrogen doses that used in birth control pills (Petitti,

2003). According our result expression of $HCO_3^-$ transporter increased under higher estrogen treatment (Figure 4.1-4.6) that resulted in higher $HCO_3^-$ and consequently fluid secretion (Figure 6.2) into the lumen. Similar effect has been reported by genistein, whereby CFTR expression was more under higher dose of genistein (Tuo, *et al.*, 2009). Under the same condition expression of NHE2 (Figure 5.3) and CA (Figure 4.7-4.10) was down regulated at higher E dose, that probably led to rapid passage of fluid into the uterine lumen and affect the ionic content(Chan, *et al.*, 2009). Increase in uterine fluid volume has been reported in ovarian hyperstimulation syndrome (OHSS) or in HSP. In OHSS animals, inhibition of CFTR resulted in alleviation of the symptoms, whereas enhanced CFTR mRNA levels reported in HSP patients (Ajonuma, *et al.*, 2005). Due to interaction between CFTR and $Cl^-/HCO_3^-$ exchanger, probably these channels together are responsible for OHSS or HSP. Furthermore increase in $HCO_3^-$ transporter that led to higher uterine fluid resulted in discrepancy in electrolyte content of uterus or rapid passage of fluid into the uterine lumen (Chan, *et al.*, 2009) would be the pathologic effect of hyper estrogenic condition on uterine content.

Hence, this results could provide a fundamental basis for the observed excessive fluid accumulation under a hyper-estrogenic state such as in the use of oral contraceptive pills (Salleh, *et al.*, 2005).

# Chapter 7

# CONCLUSION

## 7.1 Possible mechanism in the regulation of uterine fluid pH under the effect of E

Taken together, our results indicate that under the of influence E, $HCO_3^-$ transporters play a major role in making the uterine fluid alkaline via stimulation of $HCO_3^-$ secretion. The alkaline environment of uterine fluid is associated with higher secretion rate and increase in $HCO_3^-$ and $Cl^-$ content of the uterine lumen. On the other hand NHE isoform involved in higher $Na^+$ concentration. The possible mechanism could be (Figure 7.1):

CAII which is responsible for the generation of $H^+$ and $HCO_3^-$ is up regulated by 0.2E and at estrus. $HCO_3^-$ is extruded into the lumen via the apically located SLC26A6 and CFTR, in which both up-regulated by E and at estrus. In order to maintain the pHi homeostasis, $H^+$ is expelled via the basolaterally located NHE2 and NHE4 in exchange with $Na^+$. $Na^+$ is then pumped back into the blood via the basolaterally located $Na^+/K^+$ ATPase. The apically located NHE2 (and NHE4) may be involved in $Na^+$ secretion in exchange with $H^+$, thus further contributing to alkalinization of the luminal fluid by removing $H^+$. The up-regulation of a membrane bound CAXII under this condition is responsible for conversion of $H^+$ to $H_2CO_3$ that involved in $HCO_3^-$ absorption from the blood in addition to basolaterally located NBCe1 (Figure 7.1).

## 7.2 Possible mechanism in regulation of uterine fluid pH under the effect of P

Under the influence of P, increased membrane-bound CA activity and up regulation of NHE1 in addition to down regulation of other $HCO_3^-$ transporter, might be an important factor which contributes to observed decrease in the uterine fluid pH and volume. However, ENaC, well known $Na^+$ channel which up-regulated under the influence of P and involved in $Na^+$ absorption should not be neglected in the changes of uterine fluid

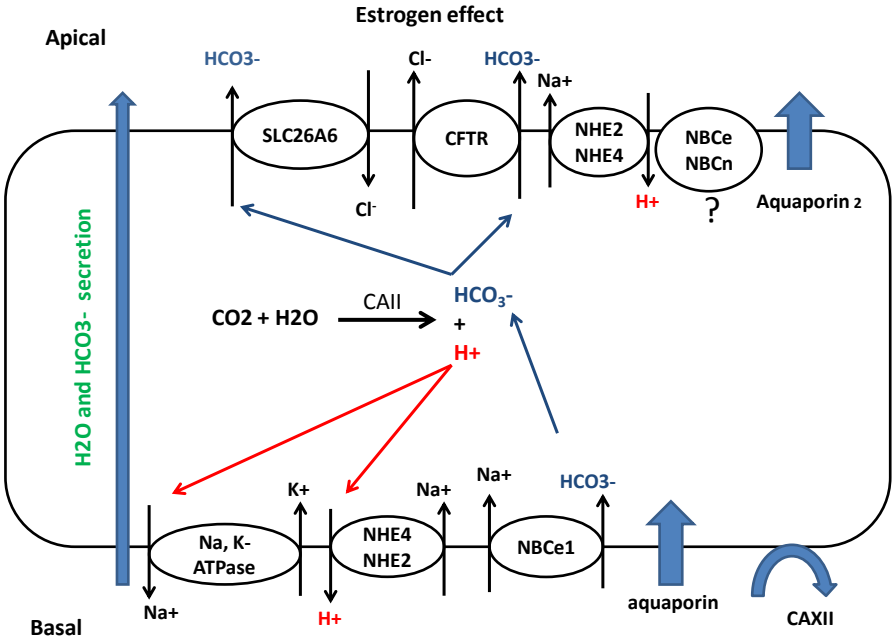



**Figure 7. 1** Proposed mechanism for regulation of uterine fluid pH under the influence of E.

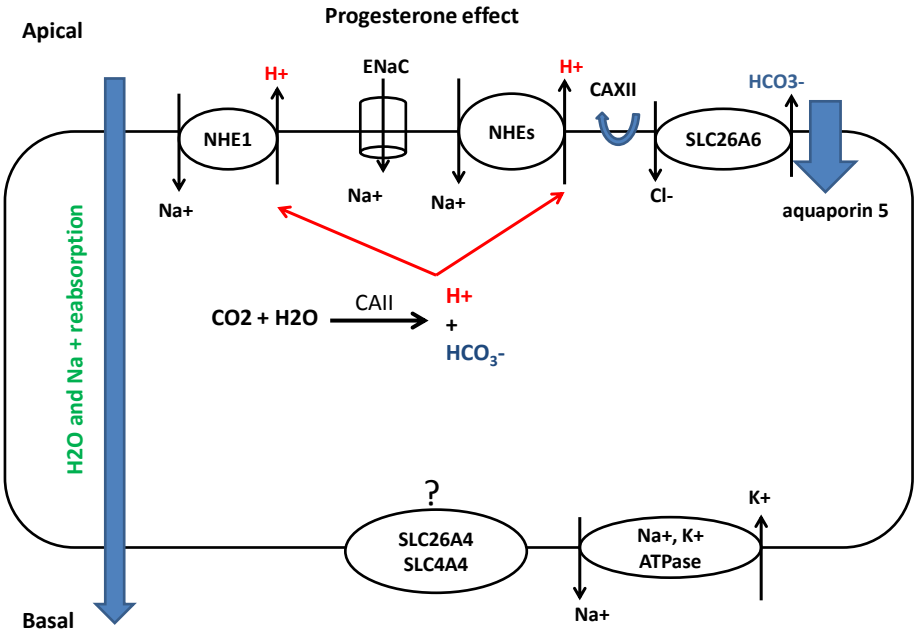



**Figure 7. 2** Possible mechanism of regulation of uterine fluid pH under the influence of P

secretion rate. Thus, the mechanism that involved in this condition could be (Figure 7.2):

CAII involves in intracellular generation of $H^+$ and $HCO_3^-$. $H^+$ will be then extruded into the lumen via the apically located NHE1. Other NHE isoforms that expressed at the apical membrane at a much lower level than NHE1 may also participate in luminal $H^+$ secretion. Apically located CAXII reabsorb $HCO_3^-$ from the lumen. $HCO_3^-$ may be expelled into the plasma via the basolaterally located SLC26A6 or other $HCO_3^-$ transporter such as SLC26A4 (Suzuki, *et al.*, 2002) in order to maintain the pHi homeostasis. $Na^+$ that accumulated in the cell (via ENaC and NHE1) will be extruded into the blood via the basolateral $Na^+/K^+$ ATPase (Figure 7.2).

## 7.3 Conclusion

1- E enhances pH of uterine luminal fluid and uterine fluid volume in a dose dependent manner.

2- E increase $Na^+$, $Cl^-$ and $HCO_3^-$ concentration of the uterine luminal fluid.

3- P reduces uterine fluid pH, volume and ionic content.

4- Alkaline environment under the influence of E is antagonized by inhibition of to $HCO_3^-$ transporter and CA.

5- E up-regulates expression of CFTR, SLC26A6, NBCe1, NHE2, NHE4, CAII and CAXII.

6- Higher E dose inhibit expression of CA and NHE2.

7- P down-regulates expression of CFTR, SLC26A6 and NBCe1 as compared to E.

8- Except CAII which expressed mainly in the uterine glandular epithelium, other transporter located in glandular and luminal epithelium.

9- CAXII and NBCe1 are mostly located on the basal membrane in luminal epithelium under the influence of E.

10- Bilateral localization of NBCe1 could be due to presence of other SLC4A4 isoform in the uterus. This one can be evaluated by application of specific antibody for different variant.

In pancreas, it has been reported that $HCO_3^-$ secretion by SLC26A6 is stimulated by CFTR through phosphorylation of the CFTR R domain by PKA (Chappe, *et al.*, 2005; Chávez, *et al.*, 2011; Simpson, *et al.*, 2005). This interaction may be mediated by NHE-RF (NHE- regulatory factor), whereby its expression up-regulated by E (Masereel, *et al.*, 2003). Thus expression of PKA and NHE-RF can be investigated to further evaluate the exact mechanism of regulation of uterine fluid pH.

Due to importance of uterine closure in implantation, expression and localization of other $HCO_3^-$ transporter at this condition need to be assessed.

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## *Appendices* ***Appendix A: Real time PCR Ct value***

| CFTR | | | | | |
|---|---|---|---|---|---|
| Sample | Ct | Sample | Ct | Sample | Ct |
| control | 31.9015 | 50E3 | 28.0268 | Ms1 | 29.1222 |
| control | 31.6286 | 50E3 | 28.3313 | Ms1 | 28.5703 |
| control | 31.674 | P1 | 31.8707 | Ms1 | 28.9428 |
| control | 31.0504 | P1 | 32.0703 | Ms2 | 28.185 |
| control | 31.1688 | P1 | 31.6384 | Ms2 | 28.1633 |
| control | 30.5102 | P1 | 31.7574 | Ms3 | 30.2277 |
| control | 30.7448 | P2 | 30.7388 | Ms3 | 29.8638 |
| control | 32.4022 | P2 | 29.9379 | Ms3 | 30.5245 |
| control | 33.0592 | P2 | 30.3265 | Ds1 | 30.0412 |
| 0.2E1 | 27.1989 | P2 | 30.0985 | Ds1 | 29.602 |
| 0.2E1 | 26.9723 | P3 | 31.4994 | Ds1 | 29.4032 |
| 0.2E1 | 26.7201 | P3 | 31.5347 | Ds1 | 29.9124 |
| 0.2E1 | 26.9093 | P3 | 32.0332 | Ds2 | 29.3987 |
| 0.2E2 | 26.0996 | E+P1 | 30.8615 | Ds2 | 28.9585 |
| 0.2E2 | 26.1226 | E+P1 | 31.1524 | Ds2 | 29.5122 |
| 0.2E2 | 25.9807 | E+P1 | 30.8919 | Ds3 | 30.22 |
| 0.2E2 | 26.2609 | E+P2 | 31.1611 | Ds3 | 29.6678 |
| 0.2E3 | 26.0046 | E+P2 | 31.42 | | |
| 0.2E3 | 25.555 | E+P2 | 30.9516 | | |
| 0.2E3 | 26.1854 | E+P3 | 32.4022 | | |
| 0.2E+vehicle | 24.7983 | E+P3 | 32.0292 | | |
| 0.2E+vehicle | 24.7218 | E+P3 | 32.4204 | | |
| 0.2E+vehicle | 24.7404 | E+P3 | 31.975 | | |
| 0.2E+vehicle | 26.7331 | Ps1 | 25.1447 | | |
| 0.2E+vehicle | 26.2812 | Ps1 | 25.0988 | | |
| 0.2E+vehicle | 25.3335 | Ps1 | undetermined | | |
| 20E1 | 26.4973 | Ps2 | 24.6482 | | |
| 20E1 | 26.5899 | Ps2 | 25.0917 | | |
| 20E1 | 26.3811 | Ps2 | 25.6074 | | |
| 20E1 | 26.4995 | Ps3 | 26.5197 | | |
| 20E2 | 25.7303 | Ps3 | 26.4474 | | |
| 20E2 | 25.4378 | Ps3 | 26.4559 | | |
| 20E2 | 25.4343 | Es1 | 27.1074 | | |
| 20E2 | 25.7835 | Es1 | 27.2524 | | |
| 20E3 | 27.5348 | Es1 | 26.9835 | | |
| 20E3 | 26.973 | Es1 | 26.9836 | | |
| 20E3 | 26.7478 | Es2 | 26.2777 | | |
| 50E1 | 26.5021 | Es2 | 26.0815 | | |
| 50E1 | 26.6329 | Es2 | 26.252 | | |
| 50E2 | 25.8623 | ES2 | 25.9633 | | |
| 50E3 | 27.9472 | ES3 | 29.9602 | | |

| SLC26A6 | | | | | |
|---|---|---|---|---|---|
| **Sample** | **Ct** | **Sample** | **Ct** | **Sample** | **Ct** |
| control | 31.1835 | 50E2 | 29.975 | Es1 | 30.3145 |
| control | 30.8251 | 50E2 | 29.9864 | Es1 | 30.0326 |
| control | 30.7587 | 50E2 | 30.1259 | Es1 | 29.5062 |
| control | 30.3858 | 50E2 | 30.5371 | Es2 | 29.5086 |
| control | 30.562 | 50E3 | 30.6219 | Es2 | 29.1185 |
| control | 30.589 | P1 | 30.869 | Es2 | 29.4152 |
| control | 30.7754 | P1 | 30.5448 | Es1 | 29.5983 |
| control | 32.677 | P2 | 30.808 | Es1 | 30.076 |
| control | 32.4306 | P2 | 30.5031 | Es1 | 30.1295 |
| 0.2E1 | 27.7949 | P2 | 30.6009 | Es3 | 30.6818 |
| 0.2E1 | 27.7141 | P2 | 30.4042 | Ms1 | 30.4693 |
| 0.2E1 | 30.7357 | P3 | 30.8102 | Ms1 | 30.4601 |
| 0.2E1 | 28.1924 | P1 | 30.769 | Ms1 | 29.9677 |
| 0.2E2 | 28.3217 | P1 | 30.6448 | Ms1 | 30.1936 |
| 0.2E2 | 28.5499 | P2 | 30.708 | Ms2 | 29.5769 |
| 0.2E2 | 28.5349 | P2 | 30.4031 | Ms2 | 30.082 |
| 0.2E2 | 27.5384 | P2 | 30.6009 | Ms2 | 29.4131 |
| 0.2E3 | 27.2079 | P2 | 30.4042 | Ms2 | 29.67 |
| 0.2E3 | 27.6286 | P3 | 30.7102 | Ms3 | 30.6521 |
| 0.2E+vehicle | 28.933 | E+P1 | 30.0978 | Ms3 | 30.91 |
| 0.2E+vehicle | 28.9941 | E+P1 | 30.8054 | Ms3 | 30.2704 |
| 0.2E+vehicle | 28.9477 | E+P1 | 30.4702 | Ds1 | 30.5535 |
| 0.2E+vehicle | 29.6745 | E+P1 | 30.7059 | Ds1 | 30.3764 |
| 0.2E+vehicle | 29.6181 | E+P2 | 30.953 | Ds1 | 30.6439 |
| 0.2E+vehicle | 29.5542 | E+P2 | 30.7587 | Ds2 | 30.5156 |
| 0.2E+vehicle | 30.5872 | E+P2 | 30.6393 | Ds2 | 29.9692 |
| 0.2E+vehicle | 29.8505 | E+P2 | 30.6065 | Ds2 | 30.6279 |
| 0.2E+vehicle | 30.4159 | E+P3 | 30.1591 | Ds2 | 30.3479 |
| 20E1 | 28.9004 | E+P3 | 29.8647 | Ds3 | 30.4281 |
| 20E1 | 29.3884 | PS1 | 29.1637 | Ds3 | 31.3658 |
| 20E1 | 28.8976 | Ps1 | 29.3241 | Ds3 | 31.2427 |
| 20E1 | 29.256 | Ps1 | 28.828 | | |
| 20E2 | 30.6818 | Ps1 | 29.3353 | | |
| 20E2 | 30.2459 | Ps2 | 28.8893 | | |
| 20E2 | 30.2136 | Ps2 | 28.8123 | | |
| 20E2 | 30.4229 | Ps2 | 29.1978 | | |
| 20E3 | 30.5814 | Ps2 | 29.0491 | | |
| 50E1 | 30.0662 | Ps3 | 29.8456 | | |
| 50E1 | 29.7083 | Ps3 | 29.9153 | | |
| 50E1 | 30.2812 | Ps3 | 29.9229 | | |
| 50E1 | 30.0201 | Es1 | 30.0156 | | |

| NBC | | | | | |
|---|---|---|---|---|---|
| **Sample** | **Ct** | **Sample** | **Ct** | **Sample** | **Ct** |
| control | 28.9334 | 50E1 | 29.8024 | Es3 | 28.8414 |
| control | 28.6953 | 50E2 | 32.1264 | Es3 | 28.9701 |
| control | 28.0809 | 50E2 | 30.8004 | Es3 | 28.5158 |
| control | 31.1386 | 50E2 | 32.6676 | Ms1 | 29.2509 |
| control | 30.0613 | 50E2 | 31.7307 | Ms1 | 27.9742 |
| control | 30.0201 | 50E3 | 29.8609 | Ms1 | 28.2524 |
| control | 29.0334 | 50E3 | 29.3987 | Ms1 | 27.9275 |
| control | 29.6953 | 50E3 | 29.6642 | Ms2 | 28.9623 |
| control | 28.0809 | P1 | 29.053 | Ms2 | 28.8536 |
| control | 30.1386 | P1 | 28.4818 | Ms3 | 29.6669 |
| control | 30.0613 | P1 | 28.3093 | Ms3 | 29.0588 |
| control | 30.0201 | P1 | 28.2577 | Ds1 | 29.2791 |
| 0.2E1 | 29.7761 | P2 | 30.2327 | Ds1 | 28.6651 |
| 0.2E1 | 29.4084 | P2 | 29.1942 | Ds1 | 28.4894 |
| 0.2E1 | 29.2151 | P3 | 31.1693 | Ds2 | 27.9746 |
| 0.2E1 | 29.3037 | P3 | 31.4261 | Ds2 | 27.8964 |
| 0.2E2 | 31.1454 | E+P1 | 27.9967 | Ds2 | 28.2347 |
| 0.2E2 | 31.2088 | E+P1 | 27.9639 | Ds3 | 28.8078 |
| 0.2E2 | 31.1943 | E+P1 | 27.6556 | | |
| 0.2E2 | 31.1726 | E+P1 | 27.7538 | | |
| 0.2E3 | 30.0175 | E+P2 | 30.0352 | | |
| 0.2E3 | 30.2067 | E+P2 | 30.2931 | | |
| 0.2E3 | 29.8915 | E+P2 | 29.9642 | | |
| 2E1 | 29.6624 | E+P2 | 29.4863 | | |
| 2E1 | 29.5196 | E+P3 | 37.4499 | | |
| 2E2 | 31.6703 | Ps1 | 28.7853 | | |
| 2E2 | 31.3923 | Ps1 | 28.4415 | | |
| 2E2 | 31.6432 | Ps1 | 28.5665 | | |
| 2E3 | 30.5415 | Ps1 | 28.7581 | | |
| 2E3 | 30.4117 | Ps2 | 29.6145 | | |
| 2E3 | 30.2287 | Ps3 | 29.7335 | | |
| 20E1 | 28.969 | Ps3 | 29.6663 | | |
| 20E1 | 28.1744 | Ps3 | 30.2403 | | |
| 20E2 | 31.0385 | Es1 | 27.6045 | | |
| 20E2 | 32.3063 | Es1 | 27.8897 | | |
| 20E3 | 28.982 | Es1 | 28.1475 | | |
| 20E3 | 28.9525 | Es1 | 27.8334 | | |
| 20E3 | 29.1722 | Es2 | 28.0321 | | |
| 50E1 | 30.0151 | Es2 | 28.4545 | | |
| 50E1 | 29.767 | Es2 | 27.8712 | | |
| 50E1 | 30.2743 | Es2 | 28.0588 | | |

| CAII | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Sample** | **Ct** | **Sample** | **Ct** | **Sample** | **Ct** | **Sample** | **Ct** |
| control | 28.2679 | 20E1 | 31.2772 | P4 | 26.8866 | Es4 | 28.721 |
| control | 28.2107 | 20E1 | 31.1867 | P4 | 26.8323 | Es4 | 28.8547 |
| control | 28.3196 | 20E1 | 31.3612 | P4 | 26.9738 | Ms1 | 26.5654 |
| control | 28.2298 | 20E1 | 31.4239 | E+P1 | 27.8114 | Ms1 | 26.1724 |
| control | 28.2394 | 20E2 | 31.4512 | E+P1 | 27.8971 | Ms2 | 26.3647 |
| control | 28.5558 | 20E2 | 31.6003 | E+P1 | 27.8293 | Ms2 | 28.9699 |
| control | 28.7825 | 20E2 | 31.5137 | E+P1 | 27.9305 | Ms2 | 28.0001 |
| control | 30.1979 | 20E2 | 31.7894 | E+P2 | 27.6075 | Ms3 | 29.8332 |
| control | 30.3265 | 20E3 | 31.9996 | E+P2 | 27.3918 | Ms3 | 29.7226 |
| control | 30.2764 | 20E3 | 31.2669 | E+P2 | 27.8193 | Ms3 | 29.4831 |
| control | 24.3881 | 20E4 | 30.7898 | E+P2 | 27.8061 | Ms4 | 28.9696 |
| control | 28.119 | 20E4 | 30.3832 | E+P3 | 24.4116 | Ms4 | 26.8632 |
| control | 27.9459 | 20E4 | 30.8577 | E+P3 | 24.6783 | Ms4 | 26.1936 |
| control | 27.7153 | 20E4 | 30.8845 | E+P3 | 24.0766 | Ms4 | 26.115 |
| 0.2E1 | 27.2391 | 50E1 | 30.0799 | E+P4 | 25.8203 | Ms4 | 25.983 |
| 0.2E1 | 27.0746 | 50E1 | 30.1009 | E+P4 | 25.0847 | Ds1 | 26.3435 |
| 0.2E1 | 27.3827 | 50E1 | 30.2181 | E+P4 | 25.3682 | Ds1 | 27.7674 |
| 0.2E1 | 27.2604 | 50E1 | 30.2676 | E+P4 | 25.3267 | Ds1 | 26.1811 |
| 0.2E2 | 26.6379 | 50E2 | 30.2733 | Ps1 | 30.9134 | Ds2 | 27.1631 |
| 0.2E2 | 26.5561 | 50E2 | 29.9926 | Ps1 | 30.7086 | Ds2 | 27.6077 |
| 0.2E2 | 26.9409 | 50E2 | 30.4742 | Ps1 | 30.9289 | Ds3 | 27.4959 |
| 0.2E2 | 26.7298 | 50E2 | 30.9928 | Ps1 | 31.7425 | Ds3 | 28.668 |
| 0.2E3 | 24.4044 | 50E3 | 30.6027 | Ps2 | 31.4734 | Ds3 | 28.4471 |
| 0.2E3 | 23.8725 | 50E3 | 31.1739 | Ps2 | 33.2987 | Ds3 | 27.8064 |
| 0.2E4 | 25.92 | 50E4 | 30.4099 | Ps2 | 32.869 | Ds4 | 29.7655 |
| 0.2E4 | 25.6138 | 50E4 | 29.8855 | Ps3 | 33.2955 | Ds4 | 29.1365 |
| 0.2E4 | 25.2992 | 50E4 | 30.2091 | Ps3 | 33.0235 | Ds4 | 29.3703 |
| 2E1 | 31.4511 | 50E4 | 30.3196 | Ps4 | 25.5769 | Ds4 | 29.1353 |
| 2E1 | 31.5556 | P1 | 28.0885 | Ps4 | 25.1413 | | |
| 2E1 | 31.5284 | P1 | 28.2547 | PS4 | 25.5641 | | |
| 2E1 | 31.643 | P1 | 28.1536 | PS4 | 25.3968 | | |
| 2E2 | 31.6621 | P1 | 28.2807 | Es1 | 30.0273 | | |
| 2E2 | 31.8508 | P2 | 27.8672 | Es1 | 29.3934 | | |
| 2E2 | 31.2588 | P2 | 28.2058 | Es1 | 29.382 | | |
| 2E2 | 31.7426 | P2 | 28.0182 | Es2 | 29.3048 | | |
| 2E3 | 34.2972 | P2 | 28.5833 | Es2 | 30.1382 | | |
| 2E3 | 33.9064 | P3 | 25.9421 | Es3 | 30.7831 | | |
| 2E4 | 31.5737 | P3 | 25.8146 | Es3 | 29.9246 | | |
| 2E4 | 30.4765 | P3 | 25.0276 | Es3 | 29.6427 | | |
| 2E4 | 30.8759 | P3 | 26.4022 | Es3 | 30.9783 | | |
| 2E4 | 31.1569 | P4 | 26.3936 | Es4 | 28.4791 | | |

| CAXII | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Sample** | **Ct** | **Sample** | **Ct** | **Sample** | **Ct** | **Sample** | **Ct** |
| control | 30.2081 | 20E2 | 34.5517 | E+P4 | 28.176 | Ds5 | 30.2689 |
| control | 30.8125 | 20E2 | 34.0706 | E+P4 | 27.7224 | Ds5 | 30.2145 |
| control | 31.9892 | 20E4 | 33.26 | E+P5 | 28.0313 | | |
| control | 32.0002 | 20E4 | 32.5324 | E+P5 | 28.4701 | | |
| control | 32.0502 | 20E4 | 32.4647 | Ps1 | 26.9754 | | |
| control | 30.1542 | 20E4 | 33.1973 | Ps1 | 26.2996 | | |
| control | 29.6599 | 20E5 | 30.9109 | Ps2 | 30.9901 | | |
| control | 30.5014 | 50E1 | 31.3271 | Ps2 | 30.61 | | |
| control | 30.54 | 50E1 | 31.3421 | Ps2 | 30.6932 | | |
| control | 31.1065 | 50E1 | 31.5531 | Ps4 | 29.6364 | | |
| control | 30.891 | 50E2 | 29.9323 | Ps4 | 29.4148 | | |
| 0.2E1 | 29.3373 | 50E2 | 29.3354 | Ps4 | 28.8025 | | |
| 0.2E1 | 28.3606 | 50E2 | 30.2349 | Ps5 | 29.7825 | | |
| 0.2E1 | 28.3374 | 50E2 | 30.2347 | Ps5 | 29.7744 | | |
| 0.2E1 | 28.5341 | 50E4 | 31.6841 | Es1 | 28.9185 | | |
| 0.2E2 | 30.6091 | 50E4 | 31.9743 | Es1 | 28.4727 | | |
| 0.2E2 | 30.5731 | 50E4 | 31.5505 | Es2 | 29.4952 | | |
| 0.2E2 | 30.625 | 50E4 | 31.796 | Es2 | 29.7939 | | |
| 0.2E2 | 30.3877 | 50E5 | 31.1539 | Es2 | 29.8288 | | |
| 0.2E4 | 29.0693 | P1 | 29.6102 | Es4 | 28.5944 | | |
| 0.2E4 | 29.0233 | P1 | 29.5497 | Es4 | 28.3899 | | |
| 0.2E4 | 28.9804 | P1 | 29.6752 | Es4 | 28.2577 | | |
| 0.2E4 | 28.9316 | P1 | 29.4537 | Es4 | 27.5976 | | |
| 0.2E5 | 29.7644 | P2 | 30.9331 | Es5 | 29.3155 | | |
| 0.2E5 | 29.0719 | P2 | 30.6292 | Es5 | 28.8347 | | |
| 2E1 | 31.6667 | P2 | 30.3682 | Ms1 | 25.0265 | | |
| 2E1 | 31.1326 | P2 | 30.5905 | Ms1 | 25.8499 | | |
| 2E1 | 31.9002 | P4 | 28.6689 | Ms2 | 28.0943 | | |
| 2E2 | 33.2263 | P4 | 28.4583 | Ms2 | 28.2743 | | |
| 2E2 | 33.3832 | P4 | 28.3674 | Ms4 | 25.6164 | | |
| 2E2 | 33.0165 | P4 | 29.0698 | Ms4 | 25.4701 | | |
| 2E2 | 32.7147 | P5 | 30.2735 | Ms4 | 25.331 | | |
| 2E4 | 31.9221 | P5 | 30.0563 | Ms5 | 29.0287 | | |
| 2E4 | 31.629 | E+P1 | 28.5262 | Ms5 | 29.0148 | | |
| 2E4 | 31.6471 | E+P1 | 28.9006 | Ms5 | 28.8836 | | |
| 2E4 | 31.6461 | E+P1 | 28.9858 | Ms5 | 29.2481 | | |
| 2E5 | 31.5841 | E+P1 | 28.6941 | Ds1 | 26.7869 | | |
| 2E5 | 30.9218 | E+P2 | 28.3653 | Ds2 | 26.8963 | | |
| 20E1 | 32.1441 | E+P2 | 28.8986 | Ds4 | 30.7868 | | |
| 20E2 | 33.9486 | E+P2 | 28.1332 | Ds5 | 30.6949 | | |
| 20E2 | 33.7868 | E+P2 | 28.5732 | Ds5 | 30.0137 | | |

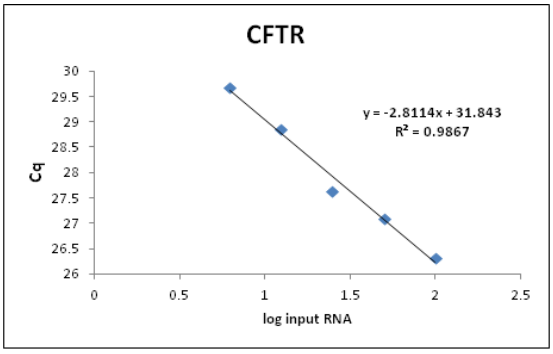
| NHE1 | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Sample** | **Ct** | **Sample** | **Ct** | **Sample** | **Ct** | **Sample** | **Ct** |
| control | 33.1894 | 20E2 | 31.3288 | E+P3 | 31.0064 | Ps2 | 30.8882 |
| control | 32.9647 | 20E2 | 31.4635 | E+P1 | 31.8185 | Ps2 | 30.6622 |
| control | 32.9364 | 20E2 | 31.3898 | Ds1 | 31.2772 | Ps2 | 30.7596 |
| control | 32.8661 | 20E3 | 31.8133 | Ds1 | 31.1674 | Ps3 | 30.7568 |
| control | 32.648 | 20E3 | 31.5256 | Ds2 | 31.3232 | | |
| control | 32.757 | 20E3 | 32.0134 | Ds2 | 31.371 | | |
| control | 32.8904 | 20E3 | 31.3695 | Ds2 | 31.1783 | | |
| control | 32.7693 | 50E1 | 32.4515 | Ds2 | 31.5101 | | |
| control | 34.3002 | 50E1 | 32.4616 | Ds3 | 31.4876 | | |
| control | 34.1813 | 50E1 | 32.1818 | Ds3 | 31.3438 | | |
| control | 33.931 | 50E1 | 32.5398 | Ds3 | 31.3245 | | |
| control | 34.592 | 50E2 | 32.3945 | Ps3 | 31.7165 | | |
| 0.2E1 | 31.3524 | 50E2 | 32.6985 | Ps3 | 31.9707 | | |
| 0.2E1 | 31.4177 | 50E2 | 32.527 | Ps3 | 31.5092 | | |
| 0.2E1 | 31.5215 | 50E2 | 32.5913 | Es1 | 30.7796 | | |
| 0.2E1 | 31.5278 | 50E3 | 33.2574 | Es1 | 30.9681 | | |
| 0.2E2 | 31.1644 | 50E3 | 32.8268 | Es1 | 31.0861 | | |
| 0.2E2 | 31.2552 | 50E3 | 32.9937 | Es2 | 31.0853 | | |
| 0.2E2 | 31.4378 | 50E3 | 33.34 | Es2 | 30.7376 | | |
| 0.2E2 | 31.5962 | P1 | 30.7122 | Es2 | 31.3899 | | |
| 0.2E3 | 32.1292 | P1 | 30.9372 | Es2 | 31.3846 | | |
| 0.2E3 | 31.9896 | P1 | 30.9861 | Es3 | 32.943 | | |
| 0.2E3 | 32.0228 | P1 | 31.0724 | Es3 | 31.9289 | | |
| 0.2E3 | 32.2199 | P2 | 30.4617 | Es3 | 34.0672 | | |
| 2E1 | 31.3532 | P2 | 30.8639 | Ms1 | 31.9721 | | |
| 2E1 | 31.3586 | P2 | 30.9978 | Ms1 | 32.3528 | | |
| 2E1 | 31.4406 | P2 | 30.8415 | Ms1 | 32.494 | | |
| 2E1 | 29.685 | P3 | 31.8462 | Ms1 | 32.4893 | | |
| 2E2 | 31.6679 | P3 | 31.8072 | Ms2 | 32.1553 | | |
| 2E2 | 31.0806 | P3 | 31.896 | Ms2 | 32.2341 | | |
| 2E2 | 31.5304 | P3 | 31.9865 | Ms2 | 32.7934 | | |
| 2E2 | 31.5097 | E+P1 | 32.2429 | Ms2 | 32.4451 | | |
| 2E3 | 31.2431 | E+P1 | 31.9739 | Ms3 | 32.6173 | | |
| 2E3 | 31.381 | E+P1 | 31.9529 | Ms3 | undetermined | | |
| 2E3 | 32.273 | E+P2 | 32.0333 | Ms3 | 32.7455 | | |
| 2E3 | 31.6992 | E+P2 | 32.1193 | Ms3 | 32.7995 | | |
| 20E1 | 31.0884 | E+P2 | 32.0645 | Ps1 | 30.2726 | | |
| 20E1 | 31.2836 | E+P2 | 32.1115 | Ps1 | 30.6041 | | |
| 20E1 | 31.0549 | E+P3 | 32.7758 | Ps1 | 30.2618 | | |
| 20E1 | 31.2398 | E+P3 | 32.8616 | Ps1 | 30.5414 | | |
| 20E2 | 31.0169 | E+P3 | 31.3622 | Ps2 | 30.8077 | | |

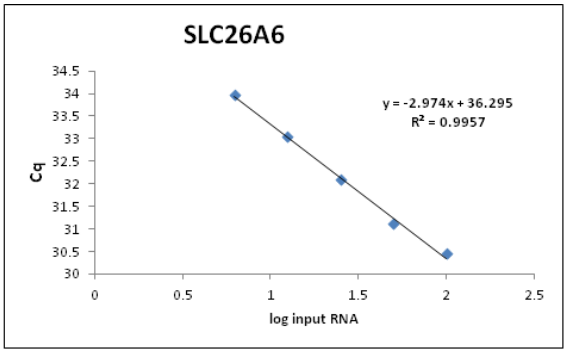
| NHE2 | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Sample** | **Ct** | **Sample** | **Ct** | **Sample** | **Ct** | **Sample** | **Ct** |
| control | 28.9538 | 2E2 | 25.3833 | Ps1 | 23.9102 | Ds3 | 25.1142 |
| control | 29.2016 | 2E2 | 25.4876 | Ps1 | 24.1631 | Ds3 | 24.4766 |
| control | 28.9961 | 2E3 | 25.9315 | Ps1 | 23.8182 | Ds3 | 24.583 |
| control | 29.1017 | 2E3 | 25.9763 | Ps1 | 23.9106 | | |
| control | 27.9248 | 2E3 | 25.8194 | Ps2 | 25.3056 | | |
| control | 27.7736 | 2E3 | 26.1783 | Ps2 | 25.2337 | | |
| control | 27.744 | 50E1 | 26.7049 | Ps2 | 24.9755 | | |
| control | 27.821 | 50E1 | 26.8698 | Ps2 | 24.8795 | | |
| control | 28.341 | 50E1 | 27.0755 | Ps3 | 25.6904 | | |
| control | 28.7064 | 50E2 | 26.3389 | Ps3 | 25.3988 | | |
| control | 28.4364 | 50E2 | 26.3049 | Es1 | 24.0915 | | |
| control | 28.7292 | 50E2 | 26.5863 | Es1 | 24.1368 | | |
| 0.2E1 | 26.4695 | 50E2 | 26.7784 | Es1 | 24.169 | | |
| 0.2E1 | 26.4219 | 50E3 | 26.8399 | Es1 | 24.3714 | | |
| 0.2E1 | 26.3692 | 50E3 | 26.6336 | Es2 | 25.5321 | | |
| 0.2E2 | 25.777 | 50E3 | 26.9937 | Es2 | 25.3591 | | |
| 0.2E2 | 25.8963 | 50E3 | 27.1758 | Es2 | 25.4399 | | |
| 0.2E2 | 25.7404 | P1 | 27.9624 | Es2 | 25.3031 | | |
| 0.2E2 | 25.8298 | P1 | 28.0712 | Es3 | 25.1658 | | |
| 0.2E3 | 26.8376 | P1 | 27.8805 | Es3 | 26.0536 | | |
| 0.2E3 | 26.8628 | P1 | 27.8948 | Es3 | 25.1214 | | |
| 0.2E3 | 26.951 | P2 | 27.1695 | Es3 | 24.9694 | | |
| 0.2E3 | 26.7595 | P2 | 27.2529 | Ms1 | 24.5594 | | |
| 20E1 | 26.4292 | P2 | 27.2443 | Ms1 | 24.6494 | | |
| 20E1 | 27.1423 | P2 | 27.0331 | Ms1 | 24.2509 | | |
| 20E1 | 26.8819 | P3 | 27.3706 | Ms1 | 24.7786 | | |
| 20E1 | 26.8099 | P3 | 27.4442 | Ms2 | 25.4297 | | |
| 20E2 | 26.4414 | P3 | 27.7007 | Ms2 | 25.5702 | | |
| 20E2 | 26.5803 | P3 | 27.6869 | Ms2 | 25.2167 | | |
| 20E2 | 26.5774 | E+P1 | 25.0707 | Ms2 | 26.1943 | | |
| 20E2 | 26.7292 | E+P1 | 24.9785 | Ms3 | 25.0447 | | |
| 20E3 | 26.9461 | E+P1 | 24.9806 | Ms3 | 25.2591 | | |
| 20E3 | 26.9373 | E+P1 | 25.08 | Ms3 | 25.3231 | | |
| 20E3 | 27.0965 | E+P2 | 27.3137 | Ms3 | 25.0592 | | |
| 20E3 | 26.9871 | E+P2 | 27.2076 | Ds1 | 23.6869 | | |
| 2E1 | 25.2248 | E+P2 | 26.4233 | Ds1 | 23.9748 | | |
| 2E1 | 25.4744 | E+P3 | 26.9729 | Ds1 | 24.353 | | |
| 2E1 | 25.6352 | E+P3 | 27.289 | Ds2 | 24.4904 | | |
| 2E1 | 25.5101 | E+P3 | 26.6391 | Ds2 | 24.5849 | | |
| 2E2 | 25.3247 | E+P3 | 26.9679 | Ds2 | 24.5829 | | |
| 2E2 | 25.5102 | Ps | 25.8575 | Ds3 | 24.4364 | | |

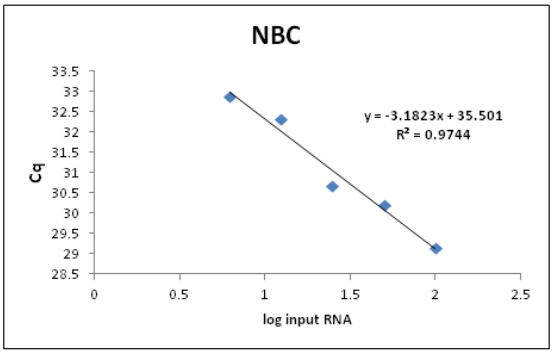
| NHE4 | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Sample** | **Ct** | **Sample** | **Ct** | **Sample** | **Ct** | **Sample** | **Ct** |
| control | 33.6234 | 20E2 | 25.926 | Ps1 | 25.4975 | Ds1 | 31.1866 |
| control | 33.5436 | 20E2 | 25.4631 | Ps1 | 25.411 | Ds1 | 30.9156 |
| control | 33.6663 | 20E2 | 25.6824 | Ps1 | 25.4669 | Ds1 | 31.1049 |
| control | 33.6782 | 20E2 | 25.0543 | Ps2 | 26.1654 | Ds1 | 31.1749 |
| control | 34.1034 | 20E3 | 26.2973 | Ps2 | 26.2551 | Ds2 | 30.743 |
| control | 33.6796 | 20E3 | 26.4499 | Ps2 | 26.1324 | Ds2 | 30.742 |
| control | 33.7697 | 20E3 | 26.5481 | Ps3 | 26.8152 | Ds2 | 30.7438 |
| control | 33.412 | 20E3 | 26.9121 | Ps3 | 26.4604 | Ds4 | 30.0759 |
| control | 35.7163 | 50E1 | 27.359 | Ps3 | 26.5356 | Ds4 | 30.753 |
| control | 35.2921 | 50E1 | 27.3596 | Ps4 | 22.8118 | Ds4 | 30.2611 |
| control | 35.9995 | 50E1 |  | Ps4 | 22.9023 | Ds4 | 29.7968 |
| control | 32.8001 | 50E2 | 26.8152 | Es1 | 27.7344 |  |  |
| 0.2E2 | 27.7431 | 50E2 | 26.8224 | Es1 | 27.0139 |  |  |
| 0.2E2 | 27.6593 | 50E2 | 26.9273 | Es1 | 26.8576 |  |  |
| 0.2E2 | 27.7824 | 50E2 | 26.9637 | Es1 | 26.9307 |  |  |
| 0.2E2 | 27.6754 | 50E3 | 27.7773 | Es2 | 27.7622 |  |  |
| 0.2E3 | 28.6306 | 50E3 | 27.6994 | Es2 | 27.4307 |  |  |
| 0.2E3 | 28.4341 | 50E3 | 27.7564 | Es2 | 27.4879 |  |  |
| 0.2E3 | 28.5148 | 50E4 | 26.2485 | Es3 | 28.9572 |  |  |
| 0.2E3 | 28.2534 | 50E4 | 26.5713 | Es3 | 28.5003 |  |  |
| 0.2E4 | 27.4236 | 50E4 | 26.4618 | Es3 | 28.2573 |  |  |
| 2E1 | 26.8517 | 50E4 | 26.2356 | Es4 | 27.6089 |  |  |
| 2E1 | 26.3874 | P2 | 33.3527 | Es4 | 27.5645 |  |  |
| 2E3 | 26.8069 | P2 | 33.7573 | Es4 | 25.8207 |  |  |
| 2E3 | 26.4578 | P2 | 34.0978 | Es4 | 25.7856 |  |  |
| 2E3 | 26.4963 | P2 | 34.4683 | Ms1 | 29.9491 |  |  |
| 2E3 | 26.516 | P3 | 34.9144 | Ms1 | 29.9597 |  |  |
| 2E3 | 27.4246 | P3 | 34.9754 | Ms1 | 30.0001 |  |  |
| 2E3 | 27.0372 | P4 | 34.5708 | Ms1 | 29.7231 |  |  |
| 2E3 | 26.9647 | P4 | 34.7531 | Ms2 |  |  |  |
| 2E3 | 27.1539 | E+P2 | 31.3012 | Ms2 | 30.7288 |  |  |
| 2E4 | 26.8172 | E+P2 | 31.1248 | Ms2 | 30.3549 |  |  |
| 2E4 | 26.435 | E+P2 | 31.49 | Ms2 | 30.9639 |  |  |
| 20E | 24.1435 | E+P2 | 31.6834 | Ms3 | 31.251 |  |  |
| 20E | 24.3839 | E+P3 | 33.5476 | Ms3 | 31.4511 |  |  |
| 20E | 24.5227 | E+P3 | 33.851 | Ms3 | 31.3559 |  |  |
| 20E | 24.5441 | E+P3 | 33.3093 | Ms3 | 31.3715 |  |  |
| 20E1 | 24.6201 | E+P4 | 30.9481 | Ms4 | 28.9033 |  |  |
| 20E1 | 24.3622 | E+P4 | 30.7745 | Ms4 | 27.5496 |  |  |
| 20E1 | 24.5361 | E+P4 | 30.3817 | Ms4 | 27.2053 |  |  |
| 20E1 | 25.8531 | Ps1 | 25.7578 | Ms4 | 27.7589 |  |  |

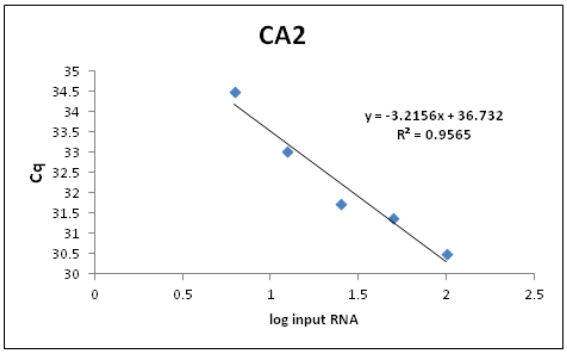
| Selection of endogenous control (housekeeping gene) for qPCR | | | | | | |
|---|---|---|---|---|---|---|
| | Hmbs | Ppia | B2m | GAPD | Hprt1 | Actb |
| control | 28.8217 | 24.5373 | 26.8878 | 25.1455 | 24.9574 | 22.2005 |
| control | 28.4682 | 24.5062 | 28.0638 | 25.1835 | 25.0702 | 22.1091 |
| control | 28.7383 | 25.8173 | 27.381 | 25.1474 | 24.8792 | 20.1237 |
| control | 29.5682 | 26.6854 | 27.9453 | 25.5378 | 24.9492 | 19.8938 |
| control | 29.5911 | 25.3983 | 28.3458 | 25.7282 | 24.9565 | 21.1436 |
| control | 29.5675 | 25.8186 | 27.381 | 25.1455 | 24.9141 | 21.2144 |
| control | 30.2205 | 25.3983 | 27.9453 | 25.1835 | 24.8981 | 22.65 |
| control | 30.2719 | 26.4503 | 26.8878 | 25.1474 | 24.5234 | 20.31 |
| control | 29.9501 | 25.2207 | 28.0638 | 25.7282 | 24.363 | 21.9518 |
| 0.2E | 28.475 | 23.9113 | 21.9523 | 23.9088 | 25.7612 | 19.6279 |
| 0.2E | 29.3247 | 22.85 | 22.4394 | 23.9762 | 25.5497 | 19.8397 |
| 0.2E | 28.9561 | 24.0476 | 22.3467 | 23.9772 | 25.5774 | 19.9223 |
| 0.2E | 29.4017 | 23.0651 | 23.5385 | 24.79 | 24.9252 | 20.2523 |
| 0.2E | 29.0716 | 23.02 | 25.0266 | 25.0797 | 25.1928 | 20.7 |
| 0.2E | 29.4017 | 23.9681 | 25.0763 | 25.126 | 25.2629 | 20.62 |
| 0.2E | 27.4533 | 22.9609 | 21.2665 | 25.92 | | 21.5401 |
| 0.2E | 27.9344 | 23.0778 | 21.4065 | 24.5437 | 24.8573 | 20.9459 |
| 0.2E | 27.4549 | 25.2074 | 21.9522 | 25.0418 | 25.3446 | 20.575 |
| 50E | 27.4533 | 22.492 | 21.2665 | 25.0588 | 25.8687 | 20.3837 |
| 50E | 27.9344 | 22.9609 | 21.4065 | 25.6819 | 25.0262 | 20.0633 |
| 50E | 27.4549 | 23.0778 | 21.9522 | 25.8245 | 25.445 | 21.2834 |
| 50E | 27.4533 | 22.9609 | 21.2665 | 25.6819 | 25.9272 | 19.71 |
| 50E | 27.9344 | 23.0778 | 21.8358 | 25.8245 | 24.5186 | 20.67 |
| P | 30.113 | 24.1907 | 28.1427 | 24.6192 | 25.2964 | 20.627 |
| P | 29.935 | 24.2252 | 28.1112 | 24.7995 | 25.2202 | 21.4676 |
| P | 29.5614 | 24.9641 | 27.6804 | 24.585 | 25.6477 | 19.9555 |
| P | 29.5549 | 24.332 | 28.7949 | 24.6921 | 25.892 | 20.1709 |
| P | 29.2282 | 24.1907 | 28.1427 | 24.8493 | 25.5331 | 20.4692 |
| P | 30.4091 | 24.2252 | 28.7248 | 25.3582 | 25.3747 | 20.627 |
| P | 29.2685 | 24.9641 | 28.8364 | 25.4805 | 25.3168 | 20.45 |
| P | 30.7544 | 24.332 | 28.6877 | 25.54 | 26.6302 | 21.12 |
| P | 30.2335 | 23.8972 | 28.7248 | 24.8493 | 25.5082 | 20.82 |
| P | 30.2793 | 24.138 | 28.8364 | 25.3582 | 25.742 | 21.312 |
| P | 30.0926 | 24.8879 | 28.6877 | 25.4805 | 25.9633 | 20.627 |
| E+P | 25.9802 | 24.6961 | 24.9756 | 24.3172 | 24.8285 | 20.11 |
| E+P | 25.6827 | 25.8511 | 23.97 | 24.7265 | 24.6262 | 22.1351 |
| E+P | 25.2595 | 22.6726 | 25.2382 | 24.6041 | 24.7402 | 22.3243 |
| E+P | 25.5081 | 25.9599 | 24.9794 | 24.6095 | 24.8552 | 19.3506 |
| E+P3 | 26.5934 | 23.6207 | 25.9438 | 25.95 | 25.5524 | 21.9262 |
| E+P | 25.8233 | 24.6762 | 25.2382 | 24.6041 | 25.4762 | 21.123 |
| E+P | 26.1657 | 24.6509 | 24.9794 | 24.6095 | 25.901 | 21.35 |
| E+P | 26.2576 | 23.234 | 25.2382 | 24.7265 | 25.645 | 20.1341 |

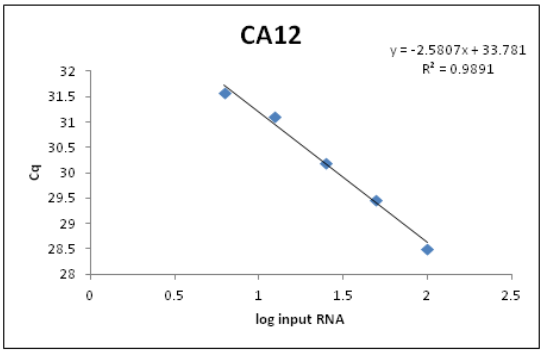
## *Appendix B: Real time assays standard curve*

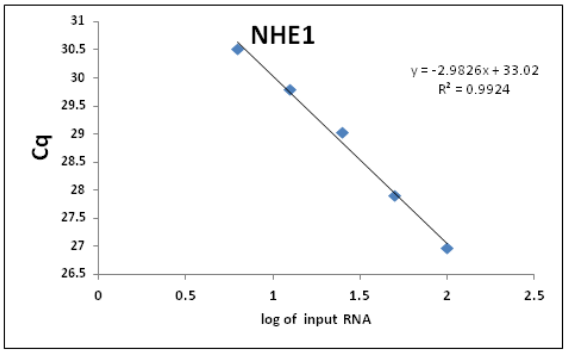


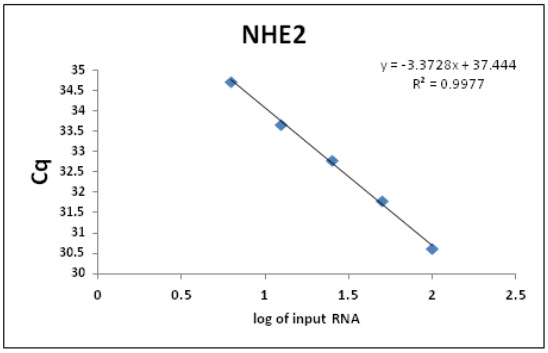



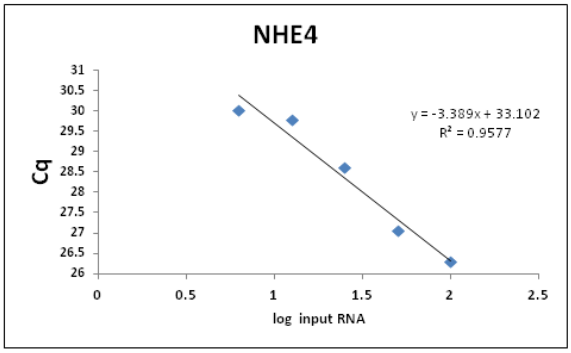



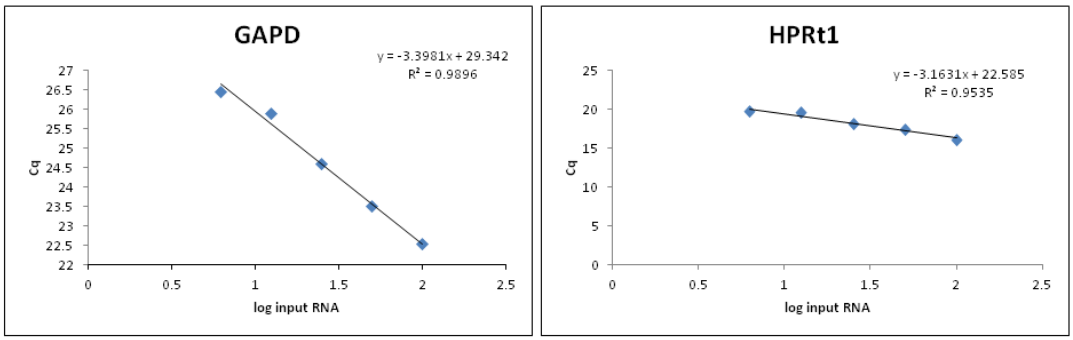






## *Appendix C: buffer preparation*

### SDS-page:

1. ***Acrylamide*** (Acrylamide, MW: 71.08 kDa and bisacrylamide, MW 154.17 kDa)

   Stock concentration of acrylamide: 30%
   Stock concentration of bisacrylamide: 0.8%
   Acrylamide (30g) and bisacrylamide (0.8g) was dissolved in double distilled water. The solutions were topped up to 100ml with double distilled water and stored in dark at 4ºC.

2. ***4 x resolving buffer*** (pH 8.8) (Tris, MW: 121.1 kDa)

   Stock solution (1.5M): 18.17g of tris was dissolved in double distilled water and volume topped up to 100ml, after adjusting of pH. The solution was stored at 4ºC.

3. ***4 x stacking buffer*** (pH 6.8) (Tris, MW: 121.1 kDa)

   Stock solution (0.5M): 3.03g of tris was dissolved in double distilled water and volume topped up to 50ml. pH was adjusted at 6.8. The solution was stored at 4ºC.

4. ***10% SDS*** (MW: 288.38 kDa)

   5g of sodium dodoceyl sulphate was dissolved in double distilled water and volume topped up to 50ml. Keep at room temperature.

5. ***10% APS*** (MW : 228.2 kDa)

   0.1g of ammonium persulphate was dissolved in double distilled water and volume topped up to 1ml.

6. ***2 x*** treatment buffer

   Stock concentration of tris: 0.125M (ph 6.8)
   Stock concentration of SDS: 4%

Stock concentration of glycerol: 20%
Stock concentration of dithiothreitol: 0.2M
Stock concentration of bromophenol blue: 0.02%
Glycerol (2ml), dithiothreitol (0.31g), bromophenol blue (0.002g), SDS (4ml of 10% SDS) and tris (2.5ml of 0.5M tris) was dissolved in double distilled water. The solution were topped up to 10ml with double distilled water and stored at 20ºC.

7. ***Tank buffer*** (Tris, MW: 121.1 kDa and glycine, MW 75.07 kDa)

Stock concentration of tris: 0.025M (pH 8.3)
Stock concentration of glycine: 0.192M
Stock concentration of SDS: 0.1%
Tris (3.03g), glycine (14.41 g) and SDS (1g) was dissolved in double distilled water. The solution were topped up to 1000L with double distilled water and stored at room temperature.

## Reagent used for Coomassie blue staining

### *Coomassie blue solution*

Stock concentration of coomassie: 0.025%
Stock concentration of methanol: 40%
Stock concentration of acetic acid: 7%
Coomassie (0.05g), methanol (80ml) and acetic acid (14ml) was topped up to 200ml with double distilled water and stored at RTºC.

### *Destain solution* Ⅰ

Stock concentration of methanol: 40%
Stock concentration of acetic acid: 7%
Methanol (80ml) and acetic acid (14ml) was topped up to 200ml with double distilled water and stored at RTºC.

### *Destain solution* Ⅱ

Stock concentration of methanol: 40%
Stock concentration of acetic acid: 7%
Methanol (10ml) and acetic acid (14ml) was topped up to 200ml with double distilled water and stored at RTºC.

## Reagent used for western-blotting

### *Towbin buffer*

Stock concentration of tris: 25mM
Stock concentration of glycine: 192mM
Stock concentration of glycine: 192mM
Tris (3.03g) and glycine (14.4g) was dissolved in double distilled water. The solution were topped up to 1L with double distilled water and stored at RT.

### *Phosphate buffer saline-tween (PBST)*

Stock concentration of bovine serum albumin (BSA): 1%
Stock concentration of tween: 0.1%
Bovine serum albumin (2.5ml) and tween (250µl) was dissolved in double distilled water. The solution were topped up to 250ml with double distilled water and stored at 4ºC.

### *Phosphate buffer saline*

Potassium phosphate (0.12g), sodium phosphate (0.72g), sodium chloride (4.0g) and potassium chloride (0.1g) was dissolved in double distilled water. The solution were topped up to 500ml with double distilled water and stored at 4ºC.

### *Perfusion buffer:*

Stock concentration (mmol/l): 110 NaCl, 143NaHCO3, 1 NaHPO4, 20 KCl, 0.8 MgSO4, 1.8 CaCl2, 5.5 glucose, 10 HEPES
NaCl (6.43g), NaHCO3 (1.2g), NaHPO4 (0.15g), KCl (1.5 g), MgSO4 (0.096g), CaCl2 (0.2g), glucose (0.99g), 0.22g all mixed and dissolved in dH2O and pH was then adjusted to 7.34.

Appendix D: Research outcomes

## Publications:

**Khadijeh Gholami**, Sekaran Muniandy and Naguib Salleh, Progesterone down-regulates CFTR and SLC26A6 protein and mRNA expression in the uterus, The Journal of Biomedicine and Biotechnology, 2012, doi:10.1155/2012/596084 (If: 2.36)

**Khadijeh Gholami**, Sekaran Muniandy and Naguib Salleh, Differential expression of Na+/H+-exchanger (NHE-1, 2 & 4) proteins and mRNA in rodent's uterus under sex-steroid effect and at different phases of the oestrous cycle. Biomed Research International,2013, doi:10.1155/2013/840121

## Presentations:

**Khadijeh Gholami**, Sekaran Muniandy and Naguib Salleh The effect of sex-steroid on the expression of carbonic anhydrase in rats uterine tissue, 2nd & 3th June 2010, Malaysian society of physiology and pharmacology (Oral presentation)

**Khadijeh Gholami**, Sekaran Muniandy and Naguib Salleh, Investigating the mechanisms underlying the effect of sex steroid on the uterine fluid volume and, 25th & 26th May 2011, Malaysian society of physiology and pharmacology (Oral presentation).

**Khadijeh Gholami**, Sekaran Muniandy and Naguib Salleh. Investigating the carbonic anhydrase effect on rat uterine fluid pH**,** 12th-14th December 2011, The 16th Biological Science Graduate Congress, Singapore (Oral presentation).

**Khadijeh Gholami**, Sekaran Muniandy and Naguib Salleh Studies on the expression and functional activities of proteins involved in uterine fluid pH regulation in female rats**,** Society for reproduction conference, 9-11 July 2012, Edinburgh, UK. (Oral presentation).